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THE BIOCHEMISTRY OF THE AMINO ACIDS

BY

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GENERAL INTRODUCTION

American Chemical Society Series of Scientific and Technologic Monographs

By arrangement with the Interallied Conference of Pure and Applied Chemistry, which met in London and Brussels in July, 1919, the American Chemical Society was to undertake the production and publication of Scientific and Technologic Monographs on chemical subjects. At the same time it was agreed that the National Research Council, in coöperation with the American Chemical Society and the American Physical Society, should undertake the production and publication of Critical Tables of Chemical and Physical Constants. The American Chemical Society and the National Research Council mutually agreed to care for these two fields of chemical development. The American Chemical Society named as Trustees, to make the necessary arrangements for the publication of the monographs, Charles L. Parsons, Secretary of the American Chemical Society, Washington, D. C.; John E. Teeple, Treasurer of the American Chemical Society, New York City; and Professor Gellert Alleman of Swarthmore College. The Trustees have arranged for the publication of the American Chemical Society series of (a) Scientific and (b) Technologic Monographs by the Chemical Catalog Company of New York City.

The Council, acting through the Committee on National Policy of the American Chemical Society, appointed the editors, named at the close of this introduction, to have charge of securing authors, and of considering critically the manuscripts prepared. The editors of each series will endeavor to select topics which are of current interest and authors who are recognized as authorities in their respective fields. The list of monographs thus far secured appears in the publisher's own announcement elsewhere in this volume.

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The development of knowledge in all branches of science, and especially in chemistry, has been so rapid during the last fifty years and the fields covered by this development have been so varied that it is difficult for any individual to keep in touch with the progress in branches of science outside his own specialty. In spite of the facilities for the examination of the literature given by Chemical Abstracts and such compendia as Beilstein's Handbuch der Organischen Chemie, Richter's Lexikon, Ostwald's Lehrbuch der Allgemeinen Chemie, Abegg's and Gmelin-Kraut's Handbuch der Anorganischen Chemie and the English and French Dictionaries of Chemistry, it often takes a great deal of time to coördinate the knowledge available upon a single topic. Consequently when men who have spent years in the study of important subjects are willing to coördinate their knowledge and present it in concise, readable form, they perform a service of the highest value to their fellow chemists.

It was with a clear recognition of the usefulness of reviews of this character that a Committee of the American Chemical Society recommended the publication of the two series of monographs under the auspices of the Society.

Two rather distinct purposes are to be served by these monographs. The first purpose, whose fulfilment will probably render to chemists in general the most important service, is to present the knowledge available upon the chosen topic in a readable form, intelligible to those whose activities may be along a wholly different line. Many chemists fail to realize how closely their investigations may be connected with other work which on the surface appears far afield from their own. These monographs will enable such men to form closer contact with the work of chemists in other lines of research. The second purpose is to promote research in the branch of science covered by the monograph, by furnishing a well digested survey of the progress already made in that field and by pointing out directions in which investigation needs to be extended. To facilitate the attainment of this purpose, it is intended to include extended references to the literature, which will enable anyone interested to follow up the subject in more detail. If the literature is so voluminous that a complete bibliography is impracticable, a critical selection will be made of those papers which are most important.

The publication of these books marks a distinct departure in the policy of the American Chemical Society inasmuch as it is a serious attempt to found an American chemical literature without primary regard to commercial considerations. The success of the venture will depend in large part upon the measure of coöperation which can be secured in the preparation of books dealing adequately with topics of general interest; it is earnestly hoped, therefore, that every member of the various organizations in the chemical and allied industries will recognize the importance of the enterprise and take sufficient interest to justify it.

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PREFACE

The purpose of a review of any field of knowledge is only partly served by a description of the work that has been done in that field. If no attempt is made to reconcile the results of experimental study, the review is little more than a chronologically arranged series of abstracts. Any effective attempt to reconcile experimental results requires a critical consideration of them and of the methods by which they have been obtained. Wherever two investigations along similar lines have been interpreted in a contradictory fashion, the reviewer must determine if possible whether the contradiction is only apparent, because of what appears to be a misinterpretation of one or the other series of data, or whether one or the other investigation is rendered totally or partially invalid by the use of faulty or inadequate methods of study. Of course, it may not be possible to effect a conciliation on either basis, so that the unraveling of the difficulty must wait upon further experimental inquiry. In fulfilling the most useful function of a monographic review the judgment of the reviewer must thus be exercised in appraising each investigation cited, in assessing its value and its significance.

The field of animal biochemistry and of nutritional physiology has been experiencing a rapid expansion during the last two decades, and inevitably with so many laboratories and so many investigators attacking the many new problems opened up by each extension of the horizon, a variety of investigational methods and working hypotheses are being used. If some are of limited usefulness, and if others become antiquated in the light of the newer accretions of knowledge, they should be discarded, but so frequently they are not. Those who attempt to make an accounting of the true progress of the science from time to time miss an opportunity and evade a responsibility if they do not, to the best of their ability, attempt a discrimination between facts and what possess only the appearance of facts, between warranted theories and outworn heirlooms, and between methods of study that at best can establish only a certain probability in favor of an outcome, and properly controlled experimentation. In its contemporary rapid expansion in volume of research, the science of nutrition is in greatest danger from the

complacency of its devotees, arising, quite naturally, from the apparent ease with which new truths may be uncovered. However, an accounting of progress actually made and an avoidance of a vast amount of misdirected experimentation requires quite a different attitude of mind. Criticism, if it does nothing more than shatter this complacency and arouse discussion of fundamentals, will justify itself, though in itself it may be opinionated and warped by purely personal viewpoints.

The review of any field of scientific research affords ample illustration of the human quirks in the interpretation of experimental data so well and so sympathetically described by D. Noel Paton in an address before the British Association for the Advancement of Science in 1919:

"It is so difficult to avoid forcing the interpretation which appeals to us. But it should be recognized that the evidence, when set down quite simply and without comment, should lead others to the same conclusion as that to which we have arrived. We are not justified in dressing it up in order to secure it more ready acceptance. In doing so, we cease to be scientific men and become special pleaders.

"Yet this dressing up of one's view so as to make it convincing is one of the most tempting of crimes—a crime which all of us, usually unconsciously, have doubtless committed in our time and will go on committing. And the worst of it is that the abler the exponent, the greater is the harm done. Every part of physiology affords startling examples of this. . . . It is so difficult to say, 'the evidence is inconclusive, to give the verdict of "Not proven."'. . . An attractive interpretation, boldly stated by an able advocate, is apt to seize the imagination even of a critical physiologist and to lead to an abrogation of judgment and a blind acceptance. Especially is this the case when the work is not in our own special line and when it is announced by a due flourish of trumpets and is supported by the invention of more or less incomprehensible Greek words devised by some classical colleague. Dangerous and unscientific as is this championing of one interpretation of a series of observations or experiments, it has not infrequently helped forward the advance of knowledge. It has often stimulated other workers and led to the true solution of the problem. . . ."

The review contained in the following pages of the present status of the subject of the biochemistry of the amino acids and their rôle in animal metabolism has been written in accordance with these ideas of the function of such a contribution. The authors are well aware of the dangers of passing judgment upon research covering such a wide field,

with only a small part of which first hand acquaintance has been gathered in the laboratory. Doubtless errors in judgment have been made, but much of the discussion offered may be of value by reason of the fact that it has been written by comparative outsiders, unhampered by the precedents and traditions unconsciously guiding investigation and directing interpretation in each sub-field of study. We have not been influenced by the prejudice so often expressed against "destructive criticism," believing that the pointing out of error is a service in itself, though possibly of a lower order than the correction of an error. Unfortunately, not all situations permit of this higher criticism, by reason of the incompleteness of available information, if not of the shortcomings of the reviewer.

The sections concerned with the chemistry of the amino acids deal with principles rather than with technical details and are not in any sense of the word a laboratory guide. Much material that it was intended to include in these sections has been curtailed or omitted entirely because of the unexpectedly large dimensions the book was assuming. The physiology of the amino acids has been considered broadly, not forgetting its bearing on the practical problems of animal nutrition. Attention has been almost entirely confined to the recent developments and to the present status of each subject discussed. A historical treatment of the subject is undoubtedly of great value, and its practical elimination from this book is not to be construed as a denial of this value, but, after all, the purposes to be served by a study of the development of scientific thought and method are different from the purposes of this book, *i.e.,* an appraisal of the extent of knowledge in the fields considered as well as of its limitations. It is well to know the facts of a science, since their acquirement is the goal of scientific investigation, but it is also well to consider what is not known, since in these directions future progress is to be made.

The authors take great pleasure in acknowledging the assistance of Miss M. Helen Keith in the preparation of this monograph. With her aid it has been possible to consult a large majority of all references cited, in order to insure an accurate description of the results obtained and a fairer judgment of their value and significance. Her acquaintance with the current literature and her daily contact with it in the course of her duties as a member of the staff of the agricultural experiment station, have contributed largely to the completeness of each chapter of this book, and of the bibliography appended to each. It is believed

that the usefulness of the monograph to those concerned directly with the topics discussed has been enhanced by definite reference to the literature for each fact or figure cited.

H. H. MITCHELL,
T. S. HAMILTON.

Urbana, Ill.

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THE BIOCHEMISTRY OF THE AMINO ACIDS

CHAPTER I

THE PHYSICAL AND CHEMICAL PROPERTIES OF THE AMINO ACIDS

The building stones of the protein molecule are all, with the two known exceptions of proline and hydroxyproline, alpha amino acids, *i.e.*, they contain an amino group in the alpha position to the carboxyl group. Even the exceptions, proline and hydroxyproline, may be regarded as amino acids of which the amino group has become involved in the closure of a pyrrolidine ring. The general type formula is, therefore, $R.CH(NH_2).COOH$. They may be considered derivatives of the saturated fatty acid series $C_nH_{2n+1}COOH$. The first member of this series is $H.COOH$ and the corresponding amino acid would be $NH_2.COOH$, but this amino acid has never been found as a constituent of the protein molecule and, in fact, has never been found to exist free in nature. The ammonium salt of this amino acid, however, is known.

The simplest amino acid which has been found as a constituent of the protein molecule is glycine, the amino derivative of acetic acid. The formula for glycine is $H.CH(NH_2).COOH$. It is interesting to note that this, the simplest known amino acid, was also the first to be discovered in protein hydrolytic products. It was discovered in 1820 by Braconnot,[1] who obtained it by boiling gelatin with dilute sulfuric acid, and on account of its sweet taste called it gelatin sugar.

The next member of the series $C_nH_{2n+1}COOH$ is propionic acid, $CH_3.CH_2.COOH$, and from this acid two amino derivatives may be obtained; namely, α- and β-amino propionic acids. Of these two the α-amino acid is the only one which occurs as a constituent of the protein molecule. The same is true of all other amino acids; it is only those acids which contain at least one of the amino group in the alpha position

[1] Braconnot, H., *Ann. chim. phys.*, 1820 [2], xiii, 113.

that are found in the protein molecule. α-Amino propionic acid is known as alanine. Alanine was prepared synthetically many years before it was discovered as a constituent of the protein molecule. It was discovered and named by Strecker [2] in 1850. Strecker prepared it from aldehyde ammonia by treating the latter with hydrogen cyanide and hydrolyzing the resulting aminocyanohydrin. The occurrence of alanine in proteins was first shown by Schützenberger and Bourgeois,[3] who obtained it in 1875 from among the decomposition products of silk.

In the homologous series with glycine and alanine are valine (discovered in 1856 by v. Gorup-Besanez [4]), leucine (probably discovered by Proust in 1818 [5] but named and identified as a protein hydrolytic

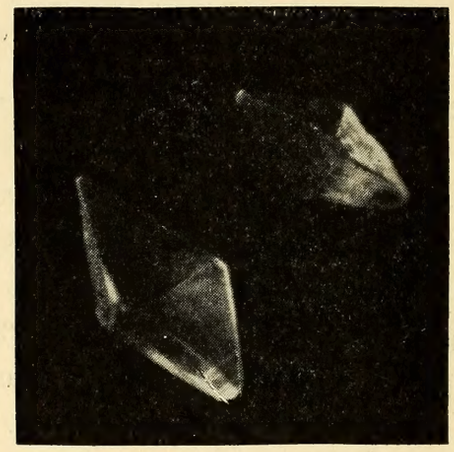


FIG. 1.—Glycine (synthetic). × 5.

Grateful acknowledgment is made to Mr. G. L. Keenan and to the *Journal of Biological Chemistry* for the use of microphotographs of the amino acids shown in Figures 1 to 7, inclusive, and 14 to 17, inclusive.

product by Braconnot [1] in 1820), and isoleucine (discovered in 1903 by F. Ehrlich [6]). These amino acids are the α-amino derivatives of isovalerianic, isocaproic, and of methyl-ethyl-propionic acids, respectively. Abderhalden and Weil [7] claimed to have discovered a fourth member of this series in norleucine or caprine, the α-amino derivative of normal caproic acid, but it cannot yet be described as a well-established amino acid constituent of proteins.

Phenylalanine (discovered by Schulze and Barbieri [8] in 1881) is, as the name suggests, the aromatic analog of alanine, while serine (discovered by Cramer [9] in 1865) and tyrosine (discovered by Liebig [10] in

[2] Strecker, A., *Ann. Chem.*, 1850, lxxv, 27.
[3] Schützenberger, P., and Bourgeois, A., *Compt. rend Acad.*, 1875, lxxxi, 1191.
[4] v. Gorup-Besanez, E., *Ann. Chem.*, 1856, xcviii, 1.
[5] Proust, M., *Ann. chim. phys.*, 1819 [2], x, 29.
[6] Ehrlich, F., *Z. Vereins deutsch. Zuckerind.*, 1903, 809.
[7] Abderhalden, E., and Weil, A., *Z. physiol. Chem.*, 1912, lxxxi, 207; 1913, lxxxviii, 272.
[8] Schulze, E., and Barbieri J., *Ber. chem. Ges.*, 1881, xiv, 1785.
[9] Cramer, E., *J. prakt. Chem.*, 1865, xcvi, 76.
[10] Liebig, J., *Ann. Chem.*, 1846, lvii, 127.

1846) are the hydroxy derivatives of alanine and phenylalanine, respectively. Harris [11] regards tyrosine as a dibasic acid since the phenolic radical titrates as a monobasic acid.

Aspartic acid (discovered by Plisson [12] in 1827) and glutamic acid (discovered by Ritthausen [13] in 1866) are monoamino derivatives of the dicarboxylic acids, succinic and glutaric, respectively. The hydroxy derivative of glutamic acid or α-amino-β-hydroxy-glutaric acid, was discovered by Dakin [14] in 1918 in hydrolyzed casein. Dakin later [15]

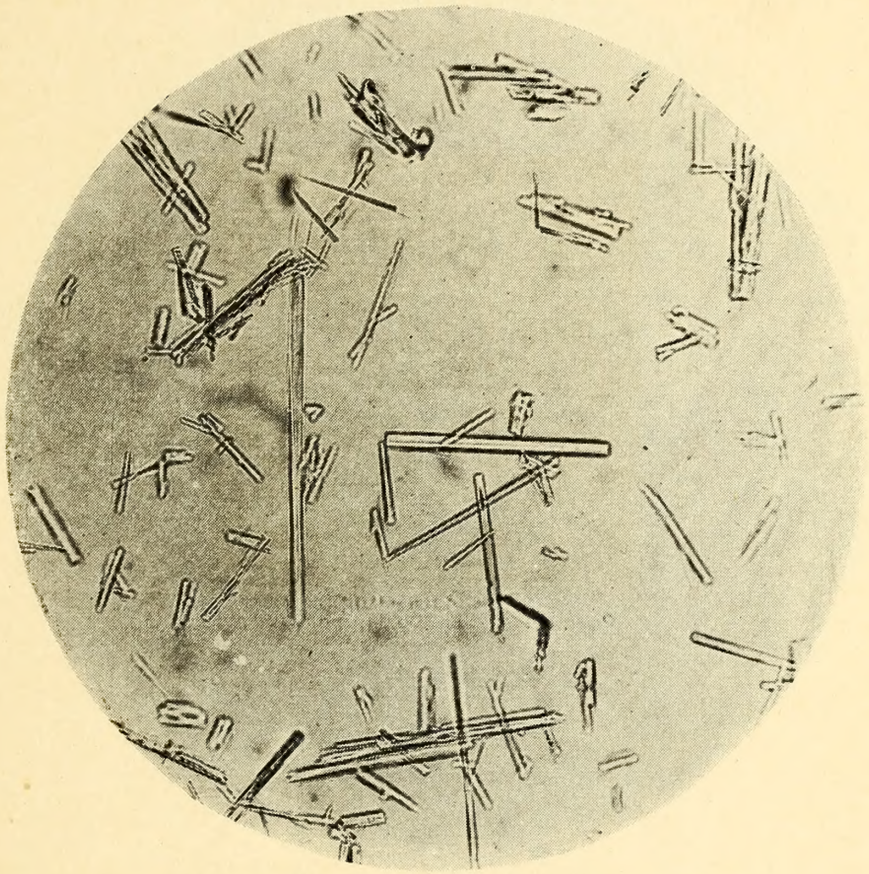


FIG. 2.—Alanine. × 230.

identified this amino acid among the hydrolytic products of gliadin and of glutenin and Jones and Johns [16] obtained it from lactalbumin. These amino acids are of special interest because of their acid character.

In comparison with aspartic and glutamic acids there are usually found in the protein molecule two acids which have two amino groups and only one carboxyl group. These are arginine (discovered by Schulze and Steiger [17] in 1866) and lysine (discovered by Drechsel [18] in 1889).

[11] Harris, L. J., *Proc. Roy. Soc. (London)*, 1923-24, B, xcv, 440.
[12] Plisson, A., *Ann. chim. phys.*, 1827 [2], xxxvi, 175.
[13] Ritthausen, H., *J. prakt. Chem.*, 1866, xcix, 6, 454.
[14] Dakin, H. D., *Biochem. J.*, 1918, xii, 290.
[15] Dakin, H. D., *Biochem. J.*, 1919, xiii, 398.
[16] Jones, D. B., and Johns, C. O., *J. Biol. Chem.*, 1921, xlviii, 347.
[17] Schulze, E., and Steiger, E., *Ber. chem. Ges.*, 1886, xix, 1177.
[18] Drechsel, E., *Ber. Verh. Kgl. Sächs. Ges. Wiss.*, 1889, xli, 117.

Arginine is δ-guanidine-α-amino-valerianic acid while lysine is α-ε-diamino-caproic acid. These two acids together with histidine (discovered by Kossel [19] in 1896), which has the composition β-imidazol-α-amino propionic acid, are frequently classed together as the hexone bases.

In addition to histidine, three more heterocyclic amino acids have been isolated from the protein molecule. These are tryptophane (dis-

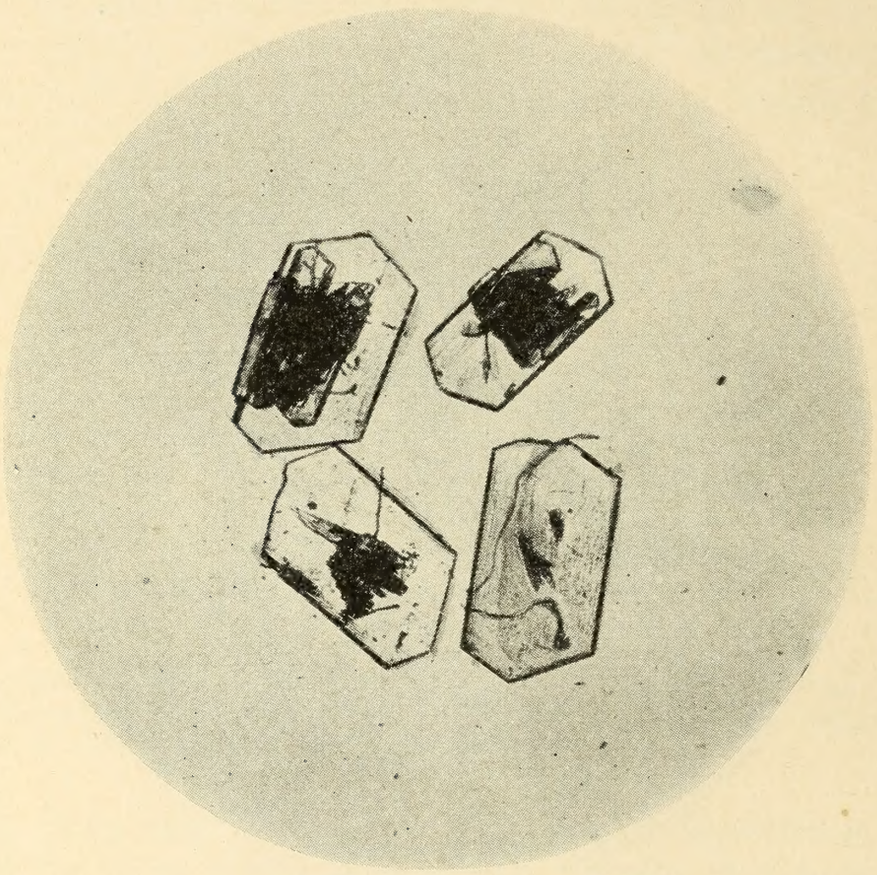


FIG. 3.—Serine. × 10.

covered by Hopkins and Cole [20] in 1902), an indole derivative of propionic acid, proline (discovered by Fischer [21] in 1901), a pyrrolidine carboxylic acid, and hydroxyproline (discovered by Fischer [22] in 1902), a hydroxy-pyrrolidine-carboxylic acid.

Two sulfur derivatives have been found as constituents of the protein molecule. These are cystine and Mueller's thio acid. Cystine was isolated in 1810 by Wollaston [23] and has the constitution, di(β-thio-α-amino propionic acid). From a study of the physical and chemical

[19] Kossel, A., *Z. physiol. Chem.*, 1896, xxii, 176.
[20] Hopkins, F. G., and Cole, S. W., *J. Physiol.*, 1901, xxvii, 418.
[21] Fischer, E., *Z. physiol. Chem.*, 1901, xxxiii, 151.
[22] Fischer, E., *Ber. chem. Ges.*, 1902, xxxv, 2660.
[23] Wollaston, W. H., *Phil. Trans. Roy. Soc.* (*London*), 1810, c, 223.

properties of cystine, Ward [24] suggests that the facts would better be explained by assuming that cystine does not possess this aliphatic structure, but that it possesses a ring structure; however, the ring structure proposed by him is impossible from the organic chemist's point of view.

Mörner,[25] Osborne and Guest,[26] Dakin,[27] Osborne,[28] Harris [29] and others have indicated that it is quite probable that there are compounds of sulfur in the protein molecule other than cystine. In 1922 Mueller [30]



FIG. 4.—Valine. × 100.

isolated from casein a new sulfur-containing amino acid, to which he gave the empirical formula $C_{11}H_{22}SN_2O_4$. Subsequent work on this amino acid by Mueller [31] indicates that the amino acid has the empirical formula $C_5H_{11}SNO_2$ instead of $C_{11}H_{22}SN_2O_4$. He offers fairly satisfactory evidence of the existence of such an amino acid in the proteins, casein, egg albumin, edestin and wool. It is present in casein to the extent of about 0.2 to 0.4 per cent. Odake [32] has recently reported the

[24] Ward, F. W., *Biochem. J.*, 1923, xvii, 898.
[25] Mörner, K. A. H., *Z. physiol. Chem.*, 1902, xxxiv, 207.
[26] Osborne, T. B., and Guest, H. H., *J. Biol. Chem.*, 1911, ix, 333.
[27] Dakin, H. D., *J. Biol. Chem.*, 1920, xliv, 499.
[28] Osborne, T. B., *J. Am. Chem. Soc.*, 1902, xxiv, 140.
[29] Harris, L. J., *Proc. Roy. Soc. (London)*, 1923, B, xciv, 426.
[30] Mueller, J. H., *Proc. Soc. Exp. Biol. Med.*, 1921-22, xix, 161.
[31] Mueller, J. H., *J. Biol. Chem.*, 1923, lvi, 157.
[32] Odake, S., *Biochem. Z.*, 1925, clxi, 446.

presence of Mueller's new sulfur-containing amino acid in an alcoholic extract of yeast. Odake found the compound to have the empirical formula $C_5H_{11}SNO_2$.

Recently Barger and Coyne [33] have shown Mueller's thio acid to have the constitution α-amino-γ-methylthiol-n-butyric acid, $CH_3.S.CH_2.CH_2.CH(NH_2).COOH$ and gave it the name methionine.

In addition to these nineteen known degradation products of protein origin, there has been described a number of related substances, found



FIG. 5.—Leucine. × 100.

among protein hydrolytic products, the proof of the presence of which in the protein molecule is still lacking. It is probable that many of these substances are compounds or mixtures of compounds formed during the treatment to which the protein was subjected in their isolation.

The presence of aminobutyric acid, which would complete the series of monoamino-carboxylic acids, was assumed in the protein molecule by Schützenberger in 1879, although proof of this is still lacking. Foreman [34] isolated a substance from casein having the empirical formula $C_4H_9NO_2$ and stated that it was α-amino isobutyric acid, although definite proof of the presence of this amino acid was insuffi-

[33] Barger, G., and Coyne, F. P., *J. Biol. Chem.*, 1928, lxxviii, Proc. p. iii.
[34] Foreman, F. W., *Biochem. Z.*, 1913, lvi, 1.

cient. Abderhalden and Weil [35] have subsequently stated that a substance of this composition had been found in varying amounts in different proteins and Abderhalden [36] has recently stated that this amino acid has been isolated and accurately identified in the products resulting from splitting lupine seed protein by fermentation. If this amino acid is present in the protein molecule it would be of special interest

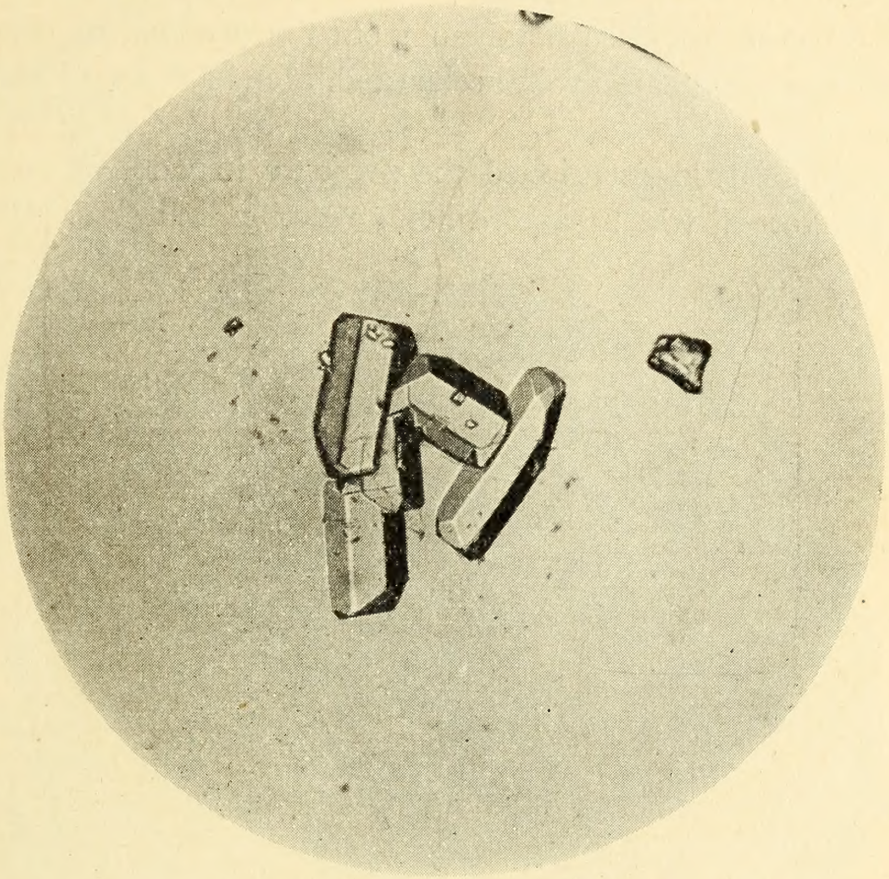


FIG. 6.—Aspartic Acid. × 80.

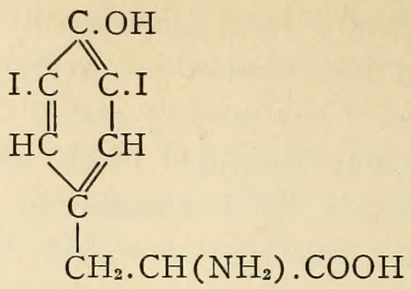
for it would be the first amino acid found in the protein molecule having a tertiary carbon atom. The formula of α-amino isobutyric acid is

$$\begin{matrix} CH_3 \\ \\ CH_3 \end{matrix}\!\!\rangle\!\!\begin{matrix} C.COOH \\ | \\ NH_2 \end{matrix}$$

This amino acid would be optically inactive.

There is definite proof that in many proteins tyrosine is found combined with iodine. This compound was found by Drechsel [37] among the hydrolytic products of the axial skeleton of a Georgonian coral. He named it Jodgorgosäure. It has the constitution 3,5-diiodotyrosine,[38]

[35] Abderhalden, E., and Weil, A., *Z. physiol. Chem.*, 1913, lxxxiv, 39; 1913, lxxxviii, 272.
[36] Abderhalden, E., "Lehrbuch der Physiologischen Chemie," Part I, Urban and Schwarzenberg, Berlin, 1923, p. 319.
[37] Drechsel, E., *Z. Biol.*, 1896, xxxiii [N.S. 15], 85.
[38] Henze, M., *Z. physiol. Chem.*, 1907, li, 64; Wheeler, H. L., and Jameson, G. S., *Am. Chem. J.*, 1905, xxxiii, 35; Wheeler, H. L., and Johns, C. O., *ibid.*, 1910, xliii, 11.



Diiodotyrosine has been obtained from the proteins of other corals and of other sea animals by Wheeler and Mendel,[39] by Oswald,[40] and by Mörner.[41]

Gortner [42] in 1911 mentioned the presence in wool of an aromatic phenolic compound, which gave Millon's reaction, but stated that it was

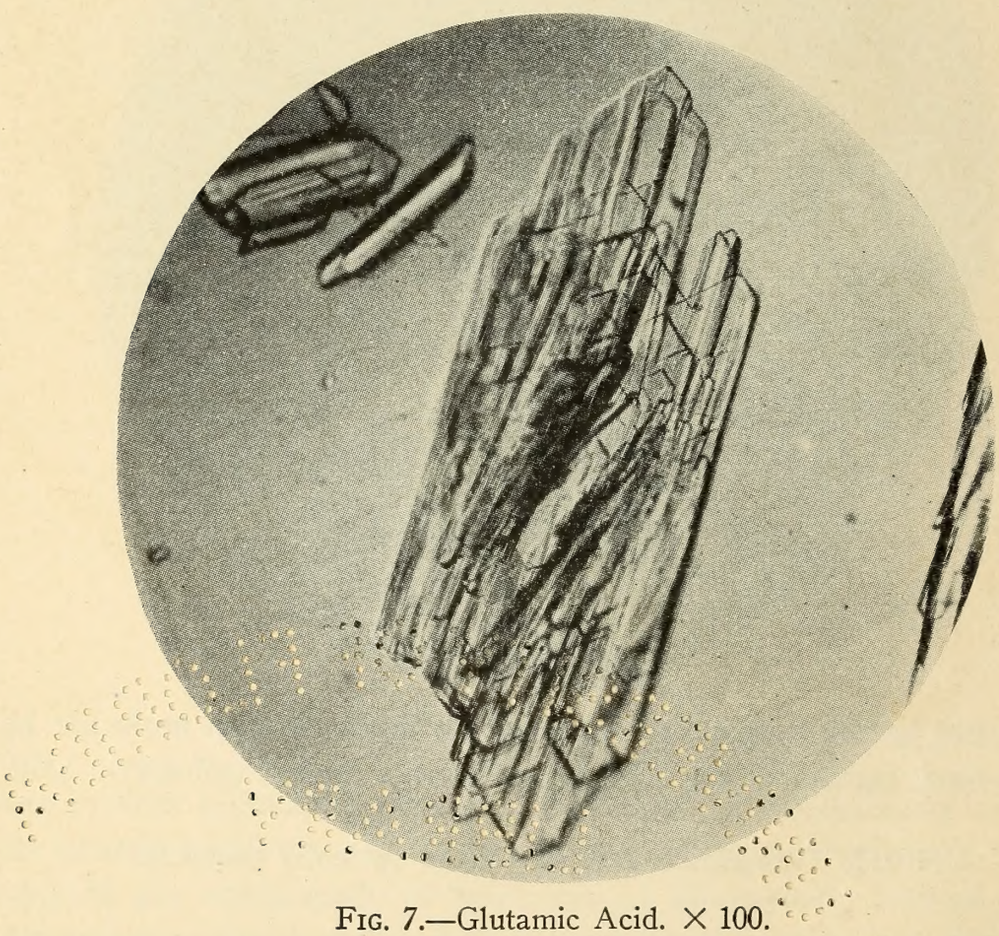


FIG. 7.—Glutamic Acid. × 100.

not tyrosine. Gortner and Hoffman [43] state that a new amino acid having the empirical formula $C_4H_{11}O_3N$ or some multiple of that has been isolated from the protein teozein, a prolamine from the seeds of teosinte (*Euchlaena Mexicana Schrad*).

[39] Wheeler, H. L., and Mendel, L. B., *J. Biol. Chem.*, 1909, vii, 1.
[40] Oswald, A., *Z. physiol. Chem.*, 1911, lxxv, 353.
[41] Mörner, C. T., *Z. physiol. Chem.*, 1907, li, 33; 1908, lv, 77, 223.
[42] Gortner, R. A., *J. Biol. Chem.*, 1911, ix, 355.
[43] Gortner, R. A., and Hoffman, W. F., *J. Am. Chem. Soc.*, 1925, xlvii, 580.

In 1921 Van Slyke and Hiller [44] mentioned an unidentified base among the hydrolytic products of gelatin. As a result of further study of this base Van Slyke and Robson [45] gave the empirical formula of the copper salt as $(C_7H_9O_4N_2)_2Cu$. The compound shows a ratio of 1 : 2 for

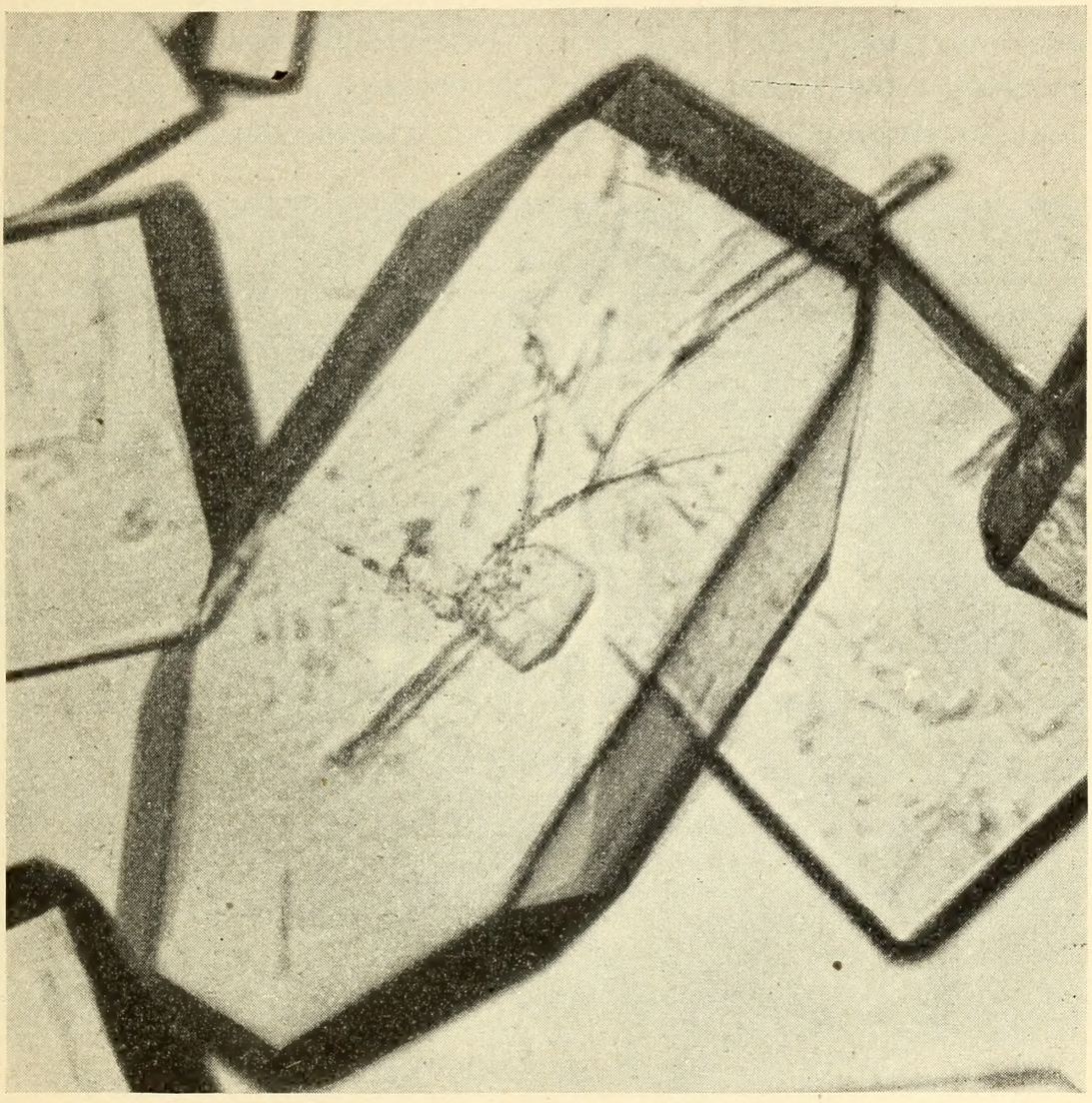


FIG. 8.—Arginine. × 120. From water.

Acknowledgment is gratefully made to Drs. H. B. Vickery and C. S. Leavenworth and to the *Journal of Biological Chemistry* for the use of microphotographs of the amino acids shown in Figures 8 to 13, inclusive.

Amino N : Total N. The behavior and analyses indicate that it may have the structure of a dihydroxy-pyrrol-alanine.

A substance of the formula $C_{11}H_{15}O_2N$ was isolated by Torquati in 1913 [46] from vetch seedlings and pods. Guggenheim [47] investigated this substance and ascertained that its elementary composition corresponded

[44] Van Slyke, D. D., and Hiller, A., *Proc. Nat. Acad. Sci.*, 1921, vii, 185.
[45] Van Slyke, D. D., and Robson, W., *Proc. Soc. Exp. Biol. Med.*, 1925, xxiii, 23.
[46] Torquati, T., *Arch. farm. sper.*, xv, 213, cited through *Chem. Zentr.*, 1913, lxxxiv (ser. 5, xvii), II, 517; *Arch. farm. sper.*, xv, 308, cited through *Chem. Zentr.*, 1913 (ser. 5, xvii), II, 518.
[47] Guggenheim, M., *Z. physiol. Chem.*, 1913, lxxxviii, 276.

more closely to the formula $C_9H_{11}O_4N$ and that its constitution was 3,4-dihydroxyphenylalanine. In properties it resembled the synthetic *dl*-dihydroxyphenylalanine described by Funk [48] and also by Stephen and Weizmann.[49]

Abderhalden and Kempe [50] in 1907, during the preparation of tryptophane by tryptic digestion of casein, observed the presence of another substance of the formula $C_{11}H_{12}N_2O_3$. It was probably an oxidation product of tryptophane. They called it oxytryptophane. Abderhalden

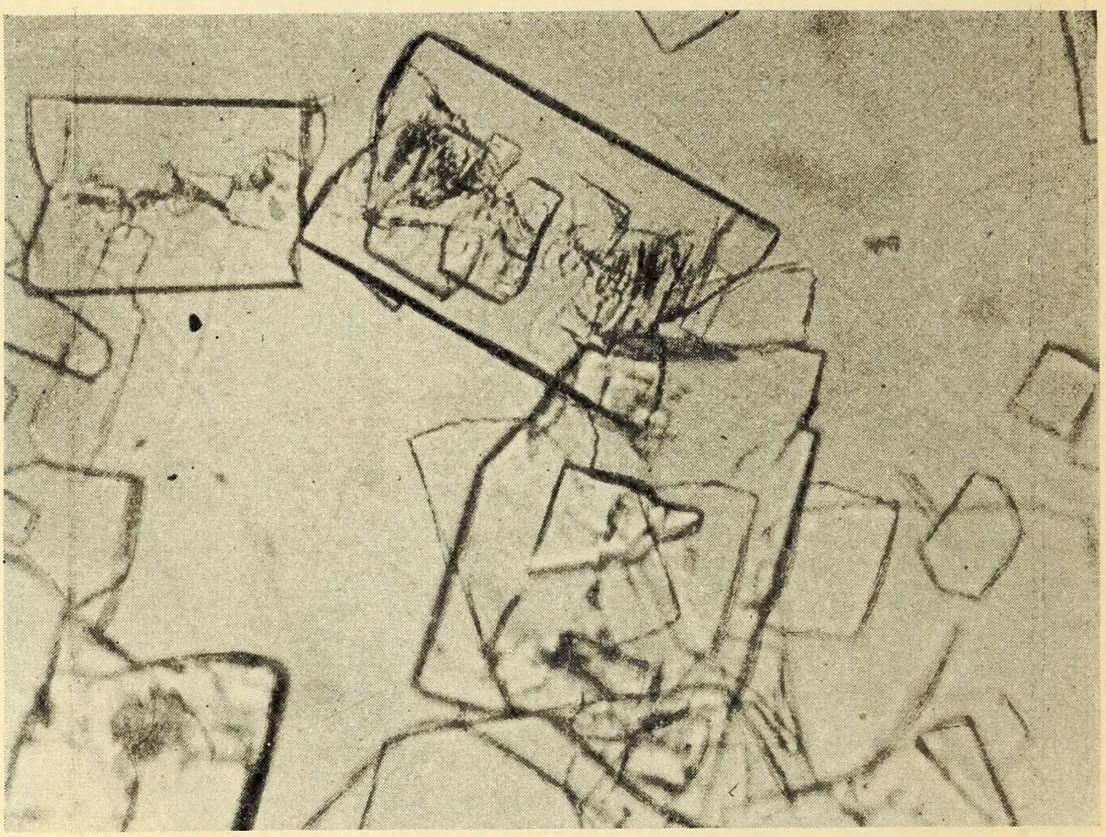


FIG. 9.—Arginine. × 150. From 66 per cent alcohol.

and Sickel [51] have reported the isolation from casein of an amino acid of the indol series having the composition $C_{11}H_{14}O_3N_2$. Their studies have indicated that this compound is a polypeptide, probably tyrosyl-proline.

Quite recently Schryver has reported the presence of three hitherto undescribed products of the hydrolysis of proteins. In 1925 [52] he reported the isolation, from isinglass, fish gelatin, and three vegetable proteins, by his "carbamate" method, of a base having the empirical

[48] Funk, C., *J. Chem. Soc., Trans.*, 1911, xcix, 554.
[49] Stephen, H., and Weizmann, C., *J. Chem. Soc., Trans.*, 1914, cv, 1152.
[50] Abderhalden, E., and Kempe, M., *Z. physiol. Chem.*, 1907, lii, 207.
[51] Abderhalden, E., and Sickel, H., *Z. physiol. Chem.*, 1924, cxxxviii, 108; 1925, cxliv, 80; 1926, cliii, 16.
[52] Schryver, S. B., Buston, H. W., and Mukherjee, D. H., *Proc. Roy. Soc. (London)*, 1925, B, xcviii, 58.

formula $C_6H_{14}O_3N_3$ and the constitution $CH_2(NH_2).CH_2.CH_2.CH(OH).CH(NH_2).COOH$, which would be hydroxy-lysine. Schryver and Buston have since reported the isolation of two additional bases, namely, protoctine [53] and *dl*-lysine.[54]

The number of amino acids contained in the protein molecule is probably limited and probably will not exceed twenty or twenty-five definite compounds. It is extremely difficult to prove that a compound found among the hydrolytic products of proteins is a naturally occur-

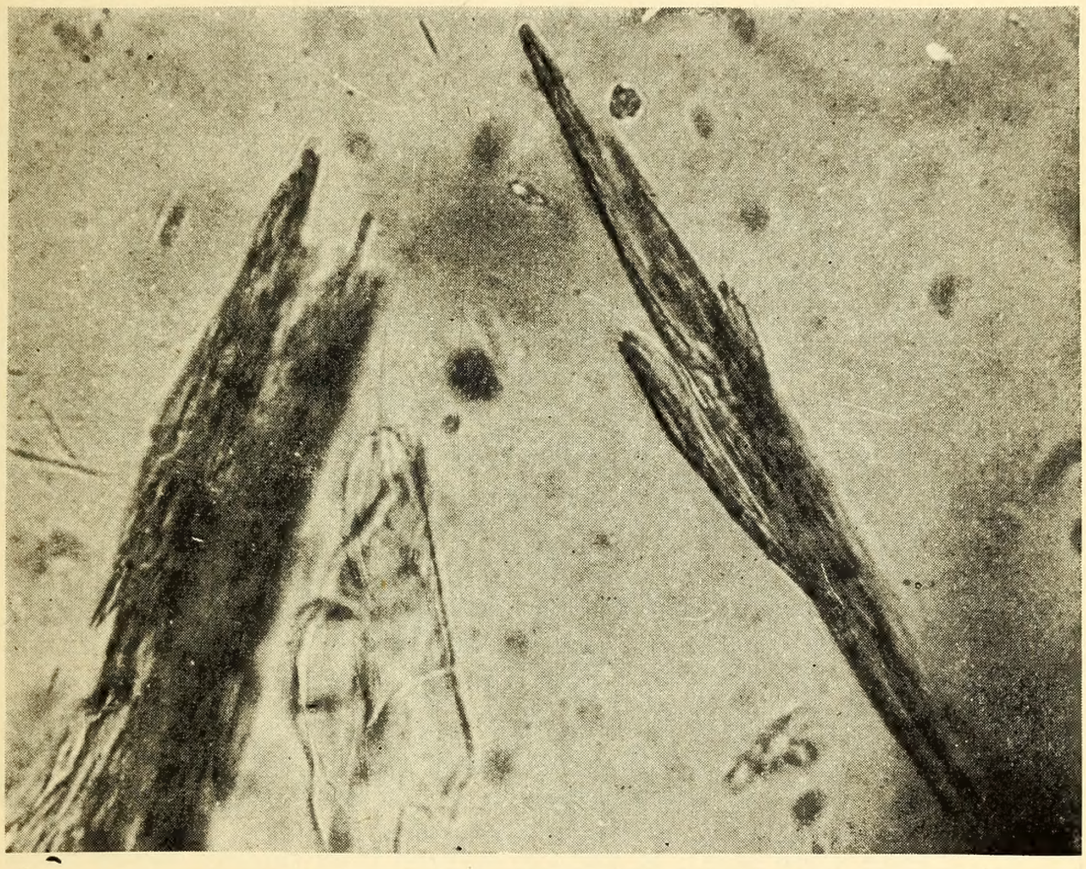


FIG. 10.—Lysine (free base). × 200. Crystallized from water.

ring constituent of the protein molecule. In the first place some dipeptides are very resistant to hydrolysis and in the second place some of the naturally occurring building stones of the protein molecule are very easily altered in the treatment to which they may be subjected during isolation.

The amino acids derived from protein hydrolysis exhibit a wide variation of structure. Some, such as alanine and leucine, possess a single amino and a single carboxyl; some, like aspartic acid, possess two carboxyl groups; and others, like lysine, two amino groups. Some, like

[53] Schryver, S. B., and Buston, H. W., *Proc. Roy Soc.* (*London*), 1926, B, xcix, 476: 1926, B, c, 360.

[54] Schryver, S. B., and Buston, H. W., *Proc. Roy. Soc.* (*London*), 1927, B, ci, 519.

serine, contain a hydroxyl radical, and cystine contains sulfur. Some are aliphatic and some are cyclic compounds. Several show, besides their amino nitrogen, nitrogen in some other combination, such as the guanidine group of arginine, the indol ring of tryptophane or the imidazole nucleus of histidine.

All the amino acids, with the exception of glycine, are optically active, and it is probable, though not yet certain, that all possess, with respect to the α-carbon atom, the same spatial configuration. All, since

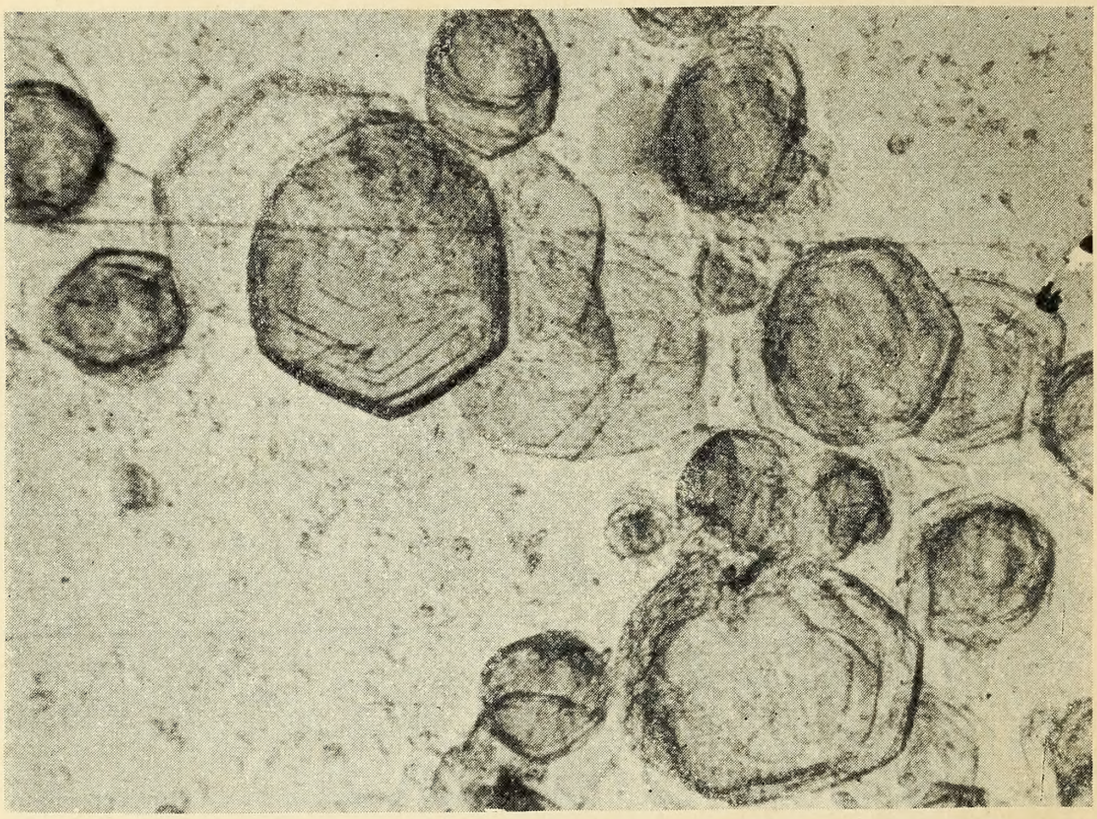


FIG. 11.—Lysine (free base). × 200. Crystallized from slightly diluted alcohol.

they contain trivalent nitrogen and a carboxyl group, are simultaneously bases and acids; in other words, all belong to the class of amphoteric electrolytes. With six exceptions all are neutral in solution. Of these six, three (arginine, histidine and lysine) are basic, while three (aspartic, glutamic and hydroxy-glutamic acids) are acidic.[55]

The amino acids are white crystalline substances and the crystal form is characteristic for each amino acid. They are readily diffusible and are not coagulable by heat. With the exception of proline they are precipitated by alcohol. They are not precipitated by ammonium sulfate or sodium chloride. They form crystalline salts with metallic bases and with mineral acids.

[55] Harris,[11] regards tyrosine as a dibasic acid since the phenolic radical titrates as a monobasic acid.

The amino acids are very weak acids and have amino groups which resemble in their properties the hydroxyl groups of the carbohydrates. They have also, in many cases, a sweet taste. Thus glycine, alanine, serine, valine, proline, hydroxyproline, tryptophane, and histidine are sweet, while leucine is tasteless and isoleucine and arginine are bitter.

The amino acids are usually classified or grouped, according to their constitution, into the following classes:

CLASSIFICATION OF THE AMINO ACIDS

I. Aliphatic Amino Acids.

A. Monoamino-monocarboxylic Acids.

1. Glycine, $C_2H_5O_2N$, or glycocoll, or amino-acetic acid.

$$\begin{array}{l} CH_2.COOH \\ | \\ NH_2 \end{array} \quad \text{or} \quad \begin{array}{l} CH_2—CO \\ | \quad | \\ NH_3—O \end{array}$$

2. *d*-Alanine, $C_3H_7O_2N$, or α-amino-propionic acid.

$$\begin{array}{l} CH_2.CH.COOH \\ \quad | \\ \quad NH_2 \end{array}$$

3. *l*-Serine, $C_3H_7O_3N$, or β-hydroxy-α-amino-propionic acid.

$$\begin{array}{l} CH_2.CH.COOH \\ | \quad | \\ OH \quad NH_2 \end{array}$$

4. *d*-Valine, $C_5H_{11}O_2N$, or α-amino-isovalerianic acid.

$$\begin{array}{l} CH_3 \\ CH_3 \end{array} > CH.CH.COOH$$
$$\qquad\qquad\quad |$$
$$\qquad\qquad NH_2$$

5. *l*-Leucine, $C_6H_{13}O_2N$, α-amino-isocaproic acid, or α-amino-isobutyl-acetic acid.

$$\begin{array}{l} CH_3 \\ CH_3 \end{array} > CH.CH_2.CH.COOH$$
$$\qquad\qquad\qquad\quad |$$
$$\qquad\qquad\qquad NH_2$$

6. *d*-Isoleucine, $C_6H_{13}O_2N$, or β-methyl-β-ethyl-α-amino-propionic acid.

$$\begin{array}{l} \quad CH_3 \\ CH_3.CH_2 \end{array} > CH.CH.COOH$$
$$\qquad\qquad\qquad\quad |$$
$$\qquad\qquad\qquad NH_2$$

B. Monoamino-dicarboxylic Acids.

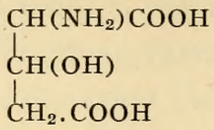
7. *l*-Aspartic acid, $C_4H_7O_4N$, or amino-succinic acid.

$$\begin{array}{l} CH_2.COOH \\ | \\ CH(NH_2).COOH \end{array}$$

8. *d*-Glutamic acid, $C_5H_9O_4N$, or α-amino-glutaric acid.

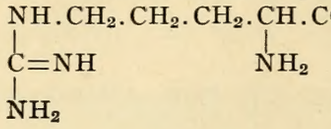
$$\begin{array}{l} CH(NH_2).COOH \\ | \\ CH_2 \\ | \\ CH_2.COOH \end{array}$$

9. *d*-Hydroxyglutamic acid, $C_5H_9O_5N$, or α-amino-β-hydroxyglutaric acid.

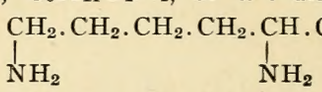


C. Diamino-monocarboxylic Acid.

10. *d*-Arginine, $C_6H_{14}O_2N_4$, or δ-guanidine-α-amino-valerianic acid.

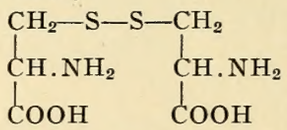


11. *d*-Lysine, $C_6H_{14}O_2N_2$, or α-ϵ-diamino-caproic acid.

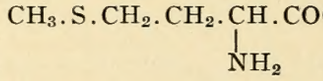


D. Sulfur-containing Amino Acid.

12. *l*-Cystine, $C_6H_{12}O_4N_2S_2$, or dicysteine, or di-(β-thio-α-amino-propionic acid).

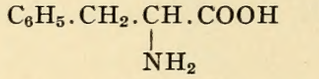


13. *l*-Methionine, $C_5H_{11}SNO_2$, or α-amino-γ-methylthiol-*n*-butyric acid.

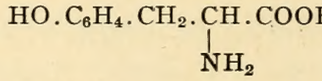


II. Aromatic Amino Acids.

14. *l*-Phenylalanine, $C_9H_{11}O_2N$, or β-phenyl-α-amino-propionic acid.

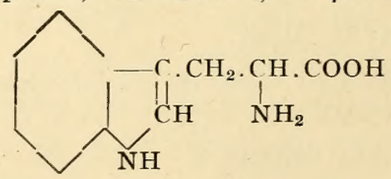


15. *l*-Tyrosine, $C_9H_{11}O_3N$, or β-para-hydroxy-phenyl-α-amino-propionic acid.

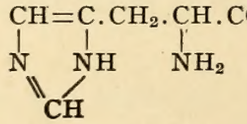


III. Heterocyclic Amino Acids.

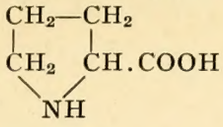
16. *l*-Tryptophane, $C_{11}H_{12}O_2N_2$, or β-indol-α-amino-propionic acid.



17. *l*-Histidine, $C_6H_9O_2N_3$, or β-imidazol-α-amino-propionic acid.



18. *l*-Proline, $C_5H_9O_2N$, or α-pyrrolidine-carboxylic acid.



19. *l*-Hydroxyproline (oxyproline), $C_5H_9O_3N$, or γ-hydroxy-α-pyrrolidine-carboxylic acid.

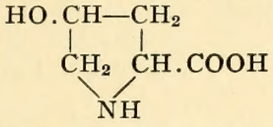


TABLE 1. *Molecular Weights, Empirical Formulas, and Percentage Composition of the Amino Acids.*

	Mol. Wt.	Empirical Formula	Percentage Composition			
			Carbon	Hydrogen	Nitrogen	Oxygen
Glycine	75.05	$C_2H_5O_2N$	31.98	6.71	18.67	42.64
d-Alanine	89.07	$C_3H_7O_2N$	40.42	7.93	15.73	35.92
l-Serine	105.07	$C_3H_7O_3N$	34.26	6.72	13.34	45.68
d-Valine	117.10	$C_5H_{11}O_2N$	51.24	9.47	11.96	27.33
l-Leucine	131.11	$C_6H_{13}O_2N$	54.97	9.99	10.69	24.35
d-Isoleucine	131.11	$C_6H_{13}O_2N$	54.97	9.99	10.69	24.35
l-Aspartic acid	133.07	$C_4H_7O_4N$	36.08	5.31	10.53	48.11
d-Glutamic acid	147.08	$C_5H_9O_4N$	40.80	6.17	9.50	43.51
d-Hydroxyglutamic acid	163.08	$C_5H_9O_5N$	36.79	5.52	8.63	49.06
d-Arginine	174.15	$C_6H_{14}O_2N_4$	41.34	8.10	32.18	18.38
d-Lysine	146.13	$C_6H_{14}O_2N_2$	49.27	9.66	19.17	21.90
l-Cystine[1]	240.26	$C_6H_{12}O_4N_2S_2$	29.97	5.03	11.65	26.66
l-Phenylalanine	165.10	$C_9H_{11}O_2N$	65.41	6.72	8.49	19.38
l-Tyrosine	181.10	$C_9H_{11}O_3N$	59.64	6.12	7.74	26.50
l-Tryptophane	204.12	$C_{11}H_{12}O_2N_2$	64.67	5.93	13.72	15.68
l-Histidine	155.10	$C_6H_9O_2N_3$	46.42	5.85	27.10	20.63
l-Proline	115.08	$C_5H_9O_2N$	52.14	7.88	12.17	27.81
l-Hydroxyproline	131.08	$C_5H_9O_3N$	45.77	6.92	10.69	36.62
l-Methionine[2]	149.16	$C_5H_{11}SNO_2$	40.23	7.43	9.39	21.45

[1] 26.69 per cent sulfur
[2] 21.50 per cent sulfur

SOLUBILITIES OF THE AMINO ACIDS

With the exception of cystine and tyrosine the amino acids are usually readily soluble in water and, with the exception of proline and hydroxyproline, insoluble in alcohol and ether. Tyrosine is only slightly soluble in cold water, but more soluble in hot water, while cystine is soluble with difficulty in both hot and cold water. Cystine, on the other hand, is very soluble. Proline and hydroxyproline are soluble in alcohol and ether.

They are all readily soluble in dilute acids and alkalies except cystine and tyrosine. Cystine does not dissolve easily in dilute ammonia and is insoluble in weak organic acids such as acetic acid. Tyrosine is only fairly soluble in weak acids and alkalies. Cystine is soluble in strong mineral acids such as hydrochloric. Leucine, while quite soluble in cold water, redissolves with much difficulty.

TABLE 2. *Physical Properties of the*

Amino Acid	Derivative	Formula	Melting Point, °C.
Glycine		$C_2H_5O_2N$	225-30[21]
	Ethyl ester hydrochloride	$C_4H_{10}O_2NCl$	144
	Copper salt	$(C_2H_4O_2N)_2Cu.H_2O$	...
	Benzoylate	$C_2H_4O_2N.C_6H_5CO$	187.5
	β-Naphthalene-sulfonate	$C_2H_4O_2N.C_{10}H_7SO_2$	159 (cor.)
	α-Naphthylisocyanate	$C_2H_4O_2N.C_{10}H_7NH.CO$	190.5-1.5
	Picrate[20]	$(C_2H_5O_2N)_2.C_6H_3O_7N_3$	202 (dec.)
	Picrolonate[1]	$C_{12}H_{13}N_5O_7$	214 (dec.)
	Phosphotungstate[2]	$(C_2H_5O_2N)_3.H_3PO_4.12WO_3.5\text{-}6H_2O$	
d-Alanine		$C_3H_7O_2N$	297 (dec.)
	Copper salt	$(C_3H_6O_2N)_2Cu$	
	Benzoylate	$C_{10}H_{11}O_3N$	150-1 (cor.)
	β-Naphthalene-sulfonate	$C_{13}H_{13}O_4NS$	122-3
	Picrolonate[1]	$C_{13}H_{15}O_8N_5$	214 (dec.)
	Phosphotungstate[2]	$(C_3H_7O_2N)_3.H_3PO_4.12WO_3.4\text{-}5H_2O$	
	α-Naphthylisocyanate[3]	$C_3H_6O_2N.C_{10}H_7NH.CO$	198
l-Serine		$C_3H_7O_3N$	228 (cor.) (dec.)
	Ethyl ester hydrochloride	$C_4H_{10}O_3NCl$	167 (cor.)
	p-Nitrobenzoylate	$C_{10}H_{10}O_6N_2$	
d-Valine		$C_5H_{11}O_2N$	315 (cor.)
	Copper salt	$(C_5H_{10}O_2N)_2Cu$	
	Phenylisocyanate	$C_{12}H_{16}O_3N_2$	147
	Picrolonate[1] †	$C_{15}H_{19}O_8N_5$	170-80
l-Leucine		$C_6H_{13}O_2N$	293-5[23]
	Copper salt	$(C_6H_{12}O_2N)_2Cu$	
	Ethyl ester hydrochloride	$C_8H_{18}O_2NCl$	134
	α-Naphthylisocyanate	$C_{17}H_{20}O_3N_2$	163.5
	β-Naphthalene-sulfonate	$C_{16}H_{19}O_4NS$	68
	Picrolonate[1] †	$C_{16}H_{21}O_7N_5$	150 (indef.)
d-Isoleucine		$C_6H_{13}O_2N$	280[23]
	Copper salt	$(C_6H_{12}O_2N)_2Cu$	
	Benzoylate	$C_{13}H_{17}O_3N$	116-7
	Benzene-sulfonate	$C_{12}H_{17}O_4NS$	149-50
	Phenylisocyanate	$C_{13}H_{18}O_3N_2$	119-20

* NOTE: The superior numbers attached to the entries in this table refer to the bibliography appended, except when they refer to degrees Centigrade. Except where indicated by specific reference number, the data contained in this table were taken from the various editions of Abderhalden's Biochemisches Handlexikon. The following abbreviations have been used:

*Amino Acids and Their Common Derivatives.**

Crystalline Structure or Color	Solubility (Figures represent grams soluble in 100 grams solvent) Water	Alcohol	Ether	Other Solvents
Large colorless prisms	$51^{20°}$	0.1	i.s.	
Long colorless needles	v.s.	d.s.	d.s.	
Blue needles (H_2O)	$0.6^{15°}$			
Rhombic prisms	$0.2^{6°}$, v.s.^{hot}	d.s.^{cold}, v.s.^{hot}	d.s.	
Elongated, pointed leaves	i.s.^{cold}, 1.1^{hot}	v.s.		
Small colorless needles	d.s. $1.8^{0°}$, v.s.^{hot}	s.^{warm}		s. alk.
Rhomboid prisms	$1.0^{20°}$			
Small whetstone-shaped crystals[6]	$4.5^{r.t.}$	14.4		
Small colorless rods	v.s.^{hot}, d.s.^{cold}	i.s.		
Long blue 6-sided leaves	v.s.			
Shiny plates	$1.2^{20°}$			
Fine clustered needles				
Long slender prisms	$1.6^{20°}$			
	$8.4^{r.t.}$	60		
Large prisms	25-33			
4- or 8-sided shiny leaves	$0.6^{25°}$			
Light yellow shiny plates				
White shiny leaves	$9.1^{16.5°}$			
Blue leaves	d.s.			v.s. MeOH
Small prisms	$1.2^{20°}$			
Colorless shiny leaves	$2^{17°(23)}$	d.s.		10.9 acetic acid
Pale blue	d.s.			
Long needles				
Long spear-like needles				
Long thin spear-like prisms	d.s.	v.s.	v.s.	
Rhombic crystals	$0.6^{20°}$			
Fine shiny leaves	$3.9^{15.5°(23)}$	i.s.^{cold}		s. acetic acid, hot
Asterisk-like leaves	$0.4^{18°}$	$0.2^{18°}$		1.8 MeOH[17]
Long shiny clear needles	i.s.^{cold}, d.s.^{hot}	v.s.	v.s.	
Colorless lances	v.s.^{hot}	v.s.	v.s.	
White shiny leaves	i.s.^{cold}, v.s.^{hot}	v.s.	v.s.	

cor. = corrected
dec. = decomposes
r.t. = room temperature
v.s. = very soluble
s. = soluble
s.s. = slightly soluble
d.s. = difficultly soluble
i.s. = insoluble
alk. = alkalies

† 19.8 per cent racemic

TABLE 2.

Amino Acid	Derivative	Formula	Melting Point, °C.
d-Isoleucine (*Continued*)			
	α-Naphthylisocyanate	$C_{17}H_{20}O_3N_2$	178
	Picrolonate [1]	$C_{16}H_{21}O_7N_5$	170
d-Norleucine		$C_6H_{13}O_2N$	285
	Copper salt	$(C_6H_{12}O_2N)_2Cu$	
l-Aspartic acid		$C_4H_7O_4N$	251 [4]
	Copper salt	$C_4H_5O_4N.Cu.4\frac{1}{2}H_2O$	
	Benzoylate	$C_{11}H_{11}O_5N$	184-5 (cor.)
	α-Naphthylisocyanate	$C_{15}H_{14}O_5N_2$	115
	Phosphotungstate [2]	$(C_4H_7O_4N)_4.2H_3PO_4.22WO_3.24H_2O$	
l-Glutamic acid		$C_5H_9O_4N$	197-8 [19]
	Hydrochloride	$C_5H_{10}O_4NCl$	193
	Copper salt	$C_5H_7O_4NCu.2\frac{1}{2}H_2O$	
	Benzoylate	$C_{12}H_{13}O_5N$	137-8
	α-Naphthylisocyanate	$C_{17}H_{16}O_5N_2$	236-7
	Phosphotungstate [5, 6]	$(C_5H_9O_4N)_3.2H_3PO_4.24WO_3$	
	Picrolonate [1]	$C_{15}H_{17}O_9N_5$	184
d-β-Hydroxy-glutamic acid [17]		$C_5H_9O_5N$	
	Silver salt [17]	$C_5H_7O_5NAg_2$	
	Copper salt [17]	$C_5H_7O_5NCu$	
d-Arginine		$C_6H_{14}O_2N_4.2H_2O$	238 (dec.) [25]
	Hydrochloride	$C_6H_{15}O_2N_4Cl$	222 (cor.) [24]
	Picrate	$C_{12}H_{17}O_9N_7.2H_2O$	217-8 (dec.) [14]
	β-Naphthalene sulfonate	$C_{16}H_{20}O_4N_4S$	87-9
	Picrolonate	$C_{16}H_{22}O_7N_8.H_2O$	225 [11], 231 [12], 237.5 [13]
	Phosphotungstate	$(C_6H_{14}O_2N_4)_3.2H_3PO_4.24WO_3.10H_2O$ [7, 8], $6H_2O$ [2]	
d-Lysine		$C_6H_{14}O_2N_2$	224 [26] (dec.)
	Hydrochloride	$C_6H_{15}O_2N_2Cl$	
	Picrate	$C_{12}H_{17}O_9N_5$	220 [15], 226 [26] (dec.)
	Picrolonate	$C_{16}H_{22}O_7N_6$	246-52 (dec.)
	Phosphotungstate [2]	$(C_6H_{14}O_2N_2)_3.2H_3PO_4.24WO_3$	
	Phenylhydantoin [15]		184-5

(*Continued*).

Crystalline Structure or Color	Solubility (Figures represent grams soluble in 100 grams solvent) Water	Alcohol	Ether	Other Solvents
Colorless needles				
Long 6-sided plates, grouped in stars	$0.6^{20°}$			
6-sided plates	d.s. $0.004^{23°\,(16)}$			
Rhombic leaves	$0.5^{16.4°}$, $5.4^{97.4°}$,	i.s.		s. salt solns.
Light blue needles	0.03^{cold}, $0.4^{100°}$ $0.4^{20°}$, $25\text{-}33^{hot}$			
Indistinct needles				
	$3.0^{r.t.}$	2.4, 400 (50%)		
Rhombic crystals	$1.0^{18°}$	0.3 (32%)		
Triclinic plates				d.s.^{cold} conc. HCl
Blue crystals	0.03^{cold}, $0.25^{100°}$			
Needles				
	s.s. v.s.	s.s.		
Thick prisms	v.s.	i.s.	i.s.	
White heavy ppt.	d.s.	d.s.	d.s.	
Blue powder	v.s.	d.s.	d.s.	
Rectangular prisms (H_2O)	v.s.	i.s.		
Small shiny plates	v.s.	d.s.^{hot} (85%)		
Long silky yellow needles	$0.5^{16°}$, $s^{100°}$			
White powder				
Yellow needles	d.s.	d.s.		
	$0.2^{r.t.\,(2)}$	$3.0^{(2)}$		
Needles	v.s.	i.s.		
Large clear crystals	v.s.			s. MeOH
Needles	$0.5^{20°}$			
	v.s.	s.		
	$3^{r.t.}$	3.6	i.s.	

TABLE 2.

Amino Acid	Derivative	Formula	Melting Point, °C.
l-Cystine		$C_6H_{12}O_4N_2S_2$	258-6 (dec.)
	Copper salt	$C_6H_{10}O_4N_2S_2Cu$	
	Benzoylate	$C_{20}H_{20}O_6N_2S_2$	180-1
	β-Naphthalene-sulfonate	$C_{26}H_{24}O_8N_2S_4$	214
	Phosphotungstate [2]	$(C_6H_{12}O_4N_2S_2)_3.2H_3PO_4.24WO_3.4\text{-}5H_2O$	
	Phenylhydantoin [13]	$C_{20}H_{18}O_4N_4S_2$	117
l-Phenylalanine		$C_9H_{11}O_2N$	283 (cor.)
	Hydrochloride	$C_9H_{12}O_2NCl$	
	Copper salt	$C_{18}H_{20}O_4N_2Cu$	
	Ethyl ester hydrochloride	$C_{11}H_{16}O_2NCl$	
	Phosphotungstate [2, 5, 9]	$(C_9H_{11}O_2N)_3.H_3PO_4.12WO_3.4H_2O$	
	Picrolonate	$C_{19}H_{19}O_7N_5$	208 (dec.)
l-Tyrosine		$C_9H_{11}O_3N$	314-8 (cor.)
	Hydrochloride	$C_9H_{12}O_3NCl$	
	Benzoylate	$C_{16}H_{15}O_4N$	165-6 (cor.)
	Copper salt	$C_{18}H_{20}O_6N_2Cu$	
	Ethyl ester hydrochloride	$C_{11}H_{16}O_3NCl$	166
	Phenylisocyanate	$C_{16}H_{16}O_4N_2$	104
	α-Naphthylisocyanate	$C_{20}H_{18}O_4N_2$	205-6
	Picrolonate [1]	$C_{19}H_{19}O_8N_5$	260 (blackens)
l-Tryptophane		$C_{11}H_{12}O_2N_2$	289 (cor.)
	Copper salt	$C_{22}H_{22}O_4N_4Cu$	
	Hydrochloride	$C_{11}H_{13}O_2N_2Cl$	251 (dec.)
	Phenylisocyanate	$C_{18}H_{17}O_3N_3$	166
	α-Naphthylisocyanate	$C_{22}H_{19}O_3N_3$	159-60
	Picrate	$C_{17}H_{15}O_9N_5$	195-6
	Picrolonate	$C_{21}H_{20}O_7N_6$	203-4
l-Methionine [23]		$C_5H_{11}SNO_2$	283 (dec.) (uncor.)
	α-Naphthol isocyanate	$C_{16}H_{18}SN_2O_3$	186 (uncor.)

(*Continued.*)

Crystalline Structure or Color	Solubility (Figures represent grams soluble in 100 grams solvent) Water	Alcohol	Ether	Other Solvents
Clear hexagonal plates	.01$^{r.t.}$	d.s.		s. acids and alk.
Blue clusters or 6-sided plates	d.s.			
Fine cauliflower-like crystals	i.s.	s.	s.s.	
Flat needles (EtOH)	d.s.	d.s.		
Fine short needles	s.s.^{cold}, v.s.^{hot}	s.s.^{cold}, v.s.^{hot} i.s.^{cold}, v.s.^{hot}		
Shiny leaves (H_2O)	3.1$^{25°}$			
Prisms				i.s. fuming HCl
Pale blue scales	i.s.			
Clear needles (EtOH+ Et_2O)	v.s.	v.s.		
	s.	s.		
Long slender rods in clusters or rect. prisms	0.3$^{20°}$			
Fine needles (H_2O)	0.05$^{25°}$	i.s.	i.s.	0.3 (0.2% HCl), 0.6 (0.2% NaOH)
Scales or plates		v.s.		
Leaves or plates (H_2O) Blue prisms	0.08^{cold}, 0.4$^{b.p.}$	i.s.	i.s.	
Silky needles (EtOH+ Et_2O)	v.s.	d.s.		
Needle clusters (H_2O)	d.s.	v.s.	v.s.	
Fine star-grouped needles				
Rods in rosettes	0.3$^{20°}$			
Hexagonal and rhombic silky leaves	s.s.^{cold}, v.s.^{hot}			
Light blue powder		s.		
				d.s. HCl
Fine needles (MeOH)	d.s.^{cold}	v.s.	v.s.	
Very small needles (EtOH)				
Red clustered needles	0.9$^{r.t.}$	v.s.	1	
Orange-red clustered needles	0.4	v.s.	v.s.	
White, hexagonal plates	s.^{cold}	s.	i.s.	
Short needles (EtOH)	i.s.	s.s.	i.s.	

TABLE 2.

Amino Acid	Derivative	Formula	Melting Point, °C.
l-Histidine		$C_6H_9O_2N_3$	277[25]
	Silver salt	$C_6H_7O_2N_3Ag_2.H_2O$	
	Monochloride	$C_6H_{10}O_2N_3Cl.H_2O$	251-2
	Dichloride	$C_6H_{11}O_2N_3Cl_2$	245
	p-Nitrobenzoylate	$C_{13}H_{12}O_5N_4$	251-2
	3-Dinaphthylene-sulfonate	$C_{26}H_{21}O_6N_3S$	149-50
	Dipicrolonate	$C_{26}H_{25}O_{12}N_{11}$	265 (dec.)
	Phosphotungstate [2]	$(C_6H_9O_2N_3)_3.2H_3PO_4.24WO_3^7,5H_2O^2$	
	Diflavianate [22]	$C_6H_9O_2N_3.2C_{10}H_6O_8N_2S.\frac{1}{2}H_2O$	251-4
l-Proline		$C_5H_9O_2N$	220-2[10]
	Copper salt	$C_{10}H_{16}O_4N_2Cu$	
	β-Naphthalene-sulfonate	$C_{15}H_{15}O_4NS$	138
	Picrate	$C_{11}H_{12}O_9N_4$	152-4[27]
	Reineckate [27]	$C_9H_{16}O_2N_7S_4Cr$	199 (uncor.) (dec.)
l-Hydroxyproline		$C_5H_9O_3N$	270
	Copper salt	$C_{10}H_{16}O_6N_2Cu$	
	Phenylisocyanate	$C_{12}H_{14}O_4N_2$	175 (dec.)
	β-Naphthalene-sulfonate	$C_{15}H_{15}O_5NS.H_2O$	91-2 (cor.)
	Hydantoin [17]	$C_6H_8O_2N_2$	165-7
	Picrate [27]	$C_{11}H_{12}O_{10}N_4$	188 (uncor.)
	Reineckate [27]	$C_{13}H_{26}O_3N_{14}S_8Cr_2.3H_2O$	248 (uncor.) (dec.)

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(*Continued*).

Crystalline Structure or Color	Solubility (Figures represent grams soluble in 100 grams solvent) Water	Alcohol	Ether	Other Solvents
Striated, brittle plates (H_2O) (100°) Vol. amorph. ppt.	v.s.	s.s.	i.s.	
Thick glassy rhombic prisms				
Large rhombic plates				
Fine needles (H_2O)	d.s.^{cold}, v.s.^{hot}	d.s.		s. acids and alk.
Small satin needles (EtOH)	i.s.	s.s.		i.s. acids; v.s. alk.
Orange-yellow crystals	0.7$^{b.p.}$			
	0.4$^{r.t.}$	8.8		
Microscopic sulfur yellow needles	s.^{hot}	d.s.		
Hydros. flat needles (EtOH +Et_2O)	v.s.	s.	i.s.	
Large dark blue plates (EtOH)	v.s.	s.		
Large flat plates (EtOHaq., H_2O)	0.8$^{b.p.}$	v.s.	d.s.	
Shiny needles (EtOH)	s.s.	s.s.^{cold}, v.s.^{hot}	s.s.	
Needles		v.s.	i.s.	
Clear platelets	v.s.	s.s.		
Deep blue needles	v.s.	i.s.		
Clear clustered plates	s.	s.	d.s.	
Thin elongated leaves (H_2O)	d.s.^{cold}, 4^{hot}	v.s.	s.	
Thick shiny needles (H_2O)	v.s.^{hot}			
Long needles	v.s.	d.s.	i.s.	
Needles	1.1$^{20°}$			

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TABLE 3. *Dissociation Constants of the Amino Acids.*

Amino Acid		K_a		K_b	T (°C.)	References [1]
Glycine		1.8×10^{-10}		2.7×10^{-12}	25	Winkelblech,[2] *Z. physik. Chem.*, 1901, xxxvi, 546.
		1.8×10^{-10}		2.6×10^{-12}	"	Harris, L. J., (*a*) *Proc. Royal Soc.* (*London*), 1923-24, B xcv, 440.
Alanine		1.9×10^{-10}		3.4 to 5.1×10^{-12}	"	Winkelblech, *loc. cit.*
		1.8×10^{-10}		2.5×10^{-12}	"	Harris, *loc. cit.* (*a*).
Valine	ca	2.0×10^{-10}		2.0×10^{-12}	"	Harris, *loc. cit.* (*a*); Harris, L. J., (*b*) *Biochem. J.*, 1923, xvii, 693.
Leucine		1.8×10^{-10}		2.3×10^{-12}	"	Winkelblech, *loc. cit.*
		1.6×10^{-11}		2.3×10^{-12}	"	Wood, J. K., *J. Chem. Soc. Trans.*, 1914, cv, 1988.
		2.5×10^{-10}		2.3×10^{-12}	"	Harris, *loc. cit.* (*a*).
		3.2×10^{-10}		2.8×10^{-12}	"	Sano, K., *Biochem. Z.*, 1926, clxviii, 14.
Aspartic acid	A_1	1.5×10^{-4}		1.2×10^{-12}	"	Winkelblech, *loc. cit.*; Lunden, *Z. physik. Chem.*, 1906, liv, 532.
	A_2	1.4×10^{-10}				
	A_1	1.1×10^{-4}		1.2×10^{-12}	..	Hopfield, Halstead, Brennan, and Acree, *Science*, 1920, lii, 614.
	A_2	1.4×10^{-10}				
	A_1	1.5×10^{-4}		1.2×10^{-12}	25	Harris, *loc. cit.* (*a*).
	A_2	1.4×10^{-10}				
		2.2×10^{-12}		1.3×10^{-12}	"	Wood, *loc. cit.*
Glutamic acid	A_1	4.1×10^{-5}		1.5×10^{-12}	"	Holmberg, *Z. physiol. Chem.*, 1908, lxii, 738.
	A_2	1.6×10^{-10}				
		1.0×10^{-11}		3.9×10^{-12}	"	Wood, *loc. cit.*
	A_1	6.3×10^{-5}		1.5×10^{-12}	"	Harris, *loc. cit.* (*a*).
	A_2	1.6×10^{-10}				
Arginine		$>1.11\times10^{-14}$	B_1	$<1.0\times10^{-7}$	"	Kanitz, (*a*) *Z. physiol. Chem.*, 1906, xlvii, 476.
			B_2	2.2×10^{-12}		
		$<1.1\times10^{-14}$	B_1	$>1.0\times10^{-7}$	"	Harris, *loc. cit.* (*a*).
			B_2	2.2×10^{-12}		
		1.4×10^{-13}	B_1	1.07×10^{-5}	20	Hunter and Borsook, *Biochem. J.*, 1924, xviii, 881.
			B_2	1.3×10^{-12}		
Lysine	ca	$1\text{-}2\times10^{-12}$	B_1	$>1.0\times10^{-7}$	25	Kanitz, *loc. cit.* (*a*) (amended by Harris, *loc. cit.* (*a*)).
			B_2	1.1×10^{-12}		
		2.0×10^{-11}	B_1	3.2×10^{-7}	"	Harris, *loc. cit.* (*a*).
			B_2	1.0×10^{-12}		
Cystine	A_1	1.4×10^{-8}	B_1	1.8×10^{-12}	"	Sano, *loc. cit.*
	A_2	1.4×10^{-10}	B_2	4.5×10^{-13}		
Phenylalanine		2.5×10^{-9}		1.3×10^{-12}	"	Kanitz, (*b*) *Archiv. Gesamt. Physiol.*, 1907, cxviii, 539.
		0.75×10^{-9}		.....	"	Harris, *loc. cit.* (*a*).

TABLE 3.—*Continued.*

Amino Acid		K_a		K_b	T(°C.)	References [1]
Tyrosine	A_1	4.0×10^{-10}		2.6×10^{-12}	25	Harris, *loc. cit.* (*a*).
	A_2	4.0×10^{-11}				
	A_1	4.0×10^{-9}		2.6×10^{-12}	"	Kanitz, *loc. cit.* (*b*).
	A_2	4.0×10^{-10}				
	A_1	7.8×10^{-10}		1.57×10^{-12}	"	Hitchcock, D. J., *J. Gen. Physiol.*, 1924, vi, 747.
	A_2	8.5×10^{-11}				
	A_1	7.9×10^{-10}		1.6×10^{-12}	"	Sano, *loc. cit.*
	A_2	4.0×10^{-11}				
Histidine		2.2×10^{-9}	B_1	5.7×10^{-9}	"	Kanitz, *loc. cit.* (*a*).
			B_2	5.0×10^{-13}	"	
		2.2×10^{-9}	B_1	5.7×10^{-9}	"	Harris, *loc. cit.* (*a*).
			B_2	5.0×10^{-13}	"	

[1] Previous compilations giving some of these data are (*a*) Landolt and Bornstein, "Physikalish—Chemische Tabellen," 1885; (*b*) *Lunden, A. physik. Chem.*, 1906, liv, 532; (*c*) Clark, "The Determination of H ions," 1922, p. 463; (*d*) Michaelis, "Die Wasserstoffionenkonzentration," 1922, 60.

[2] Winkelblech's data are taken from Harris, L. J., *Proc. Royal Soc.* (*London*), 1923-24, B, xcv, 440.

As a rule all the amino acids are soluble in 10 per cent sodium chloride, 0.2 per cent hydrochloric acid, 0.5 per cent sodium carbonate, concentrated potassium hydroxide, ammonia, and strong hydrochloric acid. Tyrosine and cystine differ from other amino acids in that they are only slightly soluble in neutral aqueous solutions. They are therefore easily obtained after hydrolysis by acids by neutralizing and concentrating the solution, when they may be crystallized out.

Tyrosine is one of the least soluble amino acids. Hitchcock [56] has recently studied its solubility at 25° C. in hydrochloric acid and in sodium hydroxide solutions varying from 0.01 to 0.05 *M*, and also in distilled water. The following table of solubilities was calculated from his investigation.

Solvent	*Grams of Tyrosine Soluble at 25° C. in 100 cc. of Solvent*
Distilled water	0.047
HCl, 0.001 *M* (approximately)	0.056
" 0.005 " "	0.074
" 0.02 " "	0.15
" 0.05 " "	0.30
NaOH, 0.001 " "	0.064
" 0.005 " "	0.135
" 0.02 " "	0.32
" 0.05 " "	0.65

While the amino acids are, for the most part, soluble in water and insoluble in alcohol, Dakin [2] has shown that most of them can be quantitatively extracted from an aqueous solution by propyl, isobutyl, and amyl alcohols.

Some physical properties, including melting point, boiling point,

[56] Hitchcock, D. I., *J. Gen. Physiol.*, 1923-24, vi, 747.

color or crystalline structure, and solubility of the amino acids and their common derivatives are tabulated in Table 2. The dissociation constants of the amino acids are given in Table 3.

GENERAL COLOR REACTIONS OF THE AMINO ACIDS

Most of the color reactions used in the detection of protein substances are dependent upon the presence of some specific amino acid or upon some specific grouping in the amino acid molecule. The only

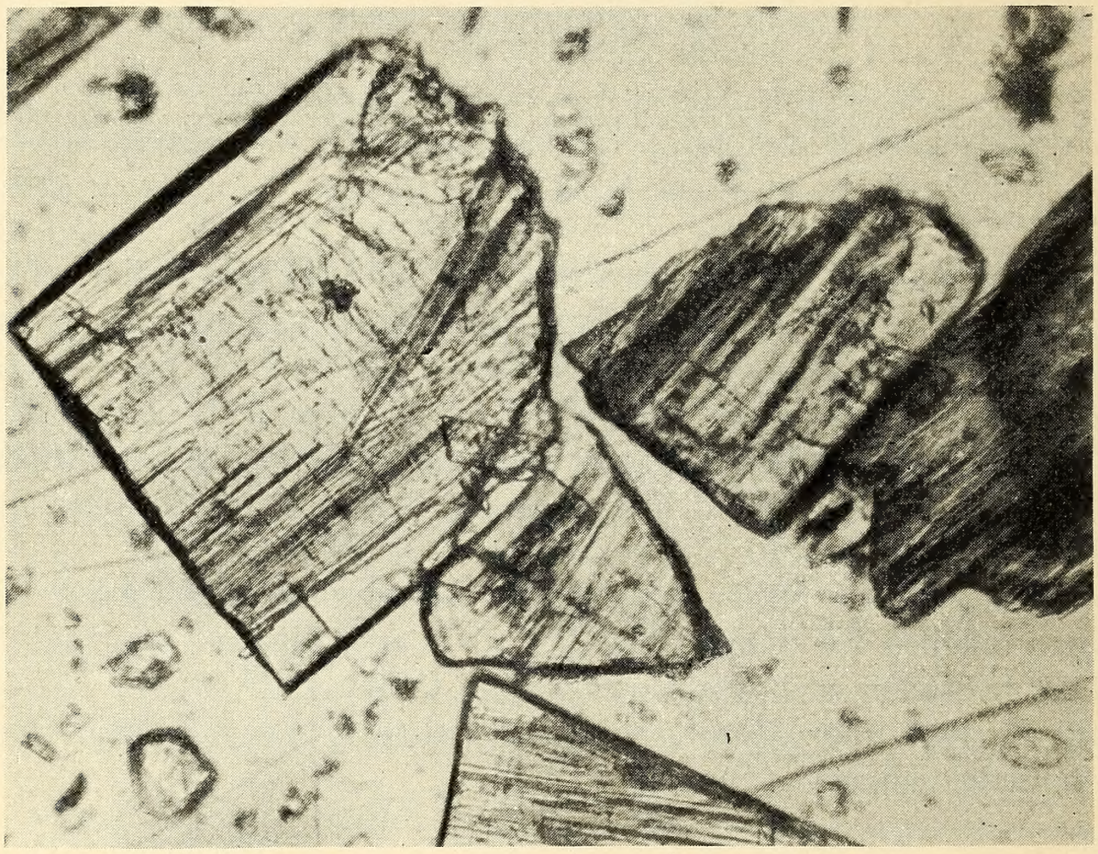


FIG. 12.—Histidine. × 125. From water.

marked exception is the biuret reaction which depends upon the constitution of the protein molecule. The peculiar grouping necessary for the biuret test seems to be at least one acid amide group and other substituted amide groups attached to adjacent carbon atoms. Since this grouping is not found in any amino acid, the amino acids do not give this test. This grouping results from the coupling of two or more amino acids; that is, it is characteristic of peptides, polypeptides, and the larger combinations of amino acids. Many other chemical substances of similar constitution also give the biuret test. Some of these substances are urea, malonamide, asparagine, etc.

The color reactions which are specific for one or more of the amino

acids have two advantages: First, they indicate the presence or absence of that particular amino acid or acids, and second, the presence of material probably of protein origin. One disadvantage of this type of reaction when used as a test for protein material is, unless the protein substance tested contains the particular amino acid or acids specific for the reagent used, a negative test does not signify the absence of protein material but only the absence of those particular amino acids. Thus a protein deficient in the particular amino acid characteristic of a color



FIG. 13.—Histidine. × 200. From 50 per cent alcohol.

reaction would escape detection. For example, Millon's reaction applied to gelatin would give a negative test, for gelatin does not contain tyrosine, the amino acid specific for this reagent.

The most important color reactions of the amino acids follow:

1. *Millon's reaction.*[57, 58] Millon's reaction consists in the development of a red color when a substance containing a monohydroxy benzene group is heated in contact with a mixture of mercuric nitrite and nitrate. This reaction is specific for tyrosine since this is the only known amino acid which contains a monohydroxy benzene group. Phenol, salicylic acid, thymol, and other organic compounds containing this

[57] Lassaigne, M., *Ann. chim. phys.*, 1830 [2], xlv, 433.
[58] Millon, E., *Compt. rend. Acad.*, 1849, xxviii, 40.

characteristic group will, of course, give the test. It is not given by a di- or tri-hydroxy phenol unless one of the hydroxyls is substituted, as in esters or ethers. Since all proteins do not contain tyrosine, gelatin and various protamines, for example, all proteins do not give Millon's test.*

Hydrogen peroxide, chlorides, and alcohol interfere with Millon's reaction, but by using an excess of the reagent (normally only a few drops of the reagent are necessary) a fairly satisfactory test may be

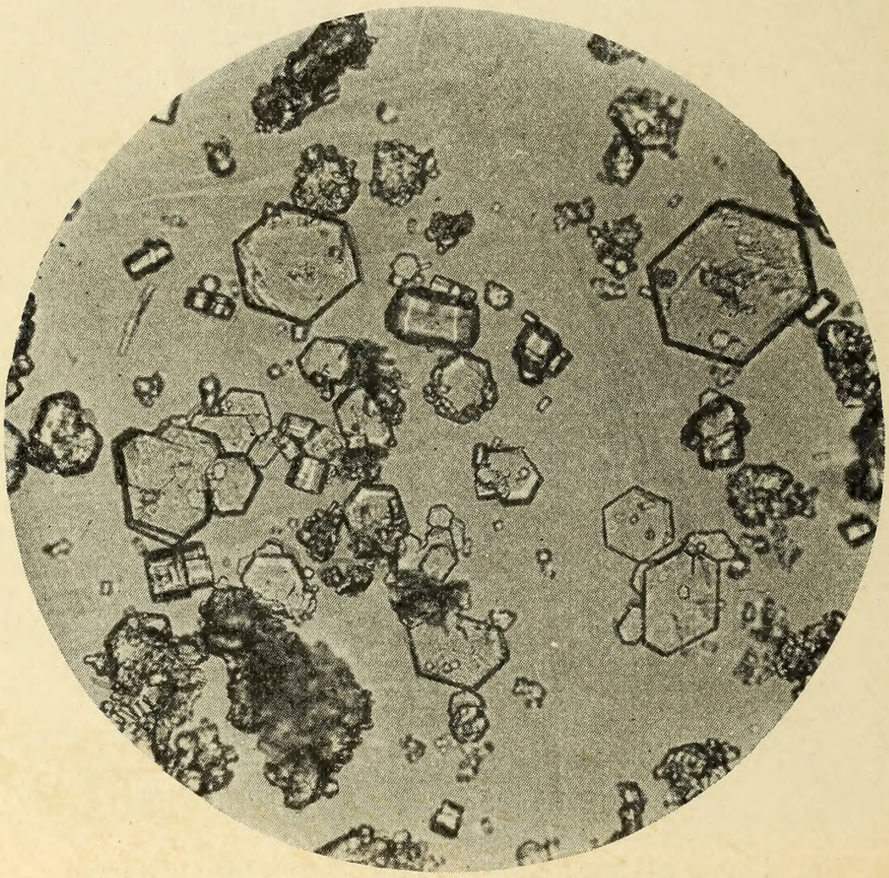


FIG. 14.—Cystine. × 100.

obtained. Large amounts of salts in a solution interfere with the test because they may precipitate the mercury and render the reagent inert. Likewise a strong alkaline solution should be neutralized inasmuch as the alkali will precipitate yellow or black oxides of mercury.

Nasse [59] recommends in place of Millon's solution, which contains a good deal of free acid, an aqueous solution of mercuric acetate, to

* This statement is questioned by Engeland [*Biochem. J.*, 19, 850 (1925)] who states that gelatin, which contains no tyrosine, and elastin, which does not contain the amino acid in sufficient amount to account for the intense color produced, give Millon's test. Engeland suspects the presence of other substances, thus far unknown as decomposition products of gelatin and elastin, which give the test.

[59] Nasse, O., *Sitzungsber. Naturforsch. Gesellsch. zu Halle,* 1879; cited through *Arch. ges. Physiol.,* 1901, lxxxiii, 361.

which at the time of using there are added a few drops of a one per cent solution of sodium or potassium nitrite.

2. *Xantho-proteic reaction.* This, as the name implies, is a yellow color produced under certain conditions. With strong nitric acid most proteins give, on heating to boiling, yellow flakes or a yellow solution. After cooling and adding ammonia or alkalies the color of the solution changes to orange-yellow.

This reaction is usually assumed to be due to the benzene nuclei in the protein molecule and should, therefore, be given by tyrosine, phenyl-

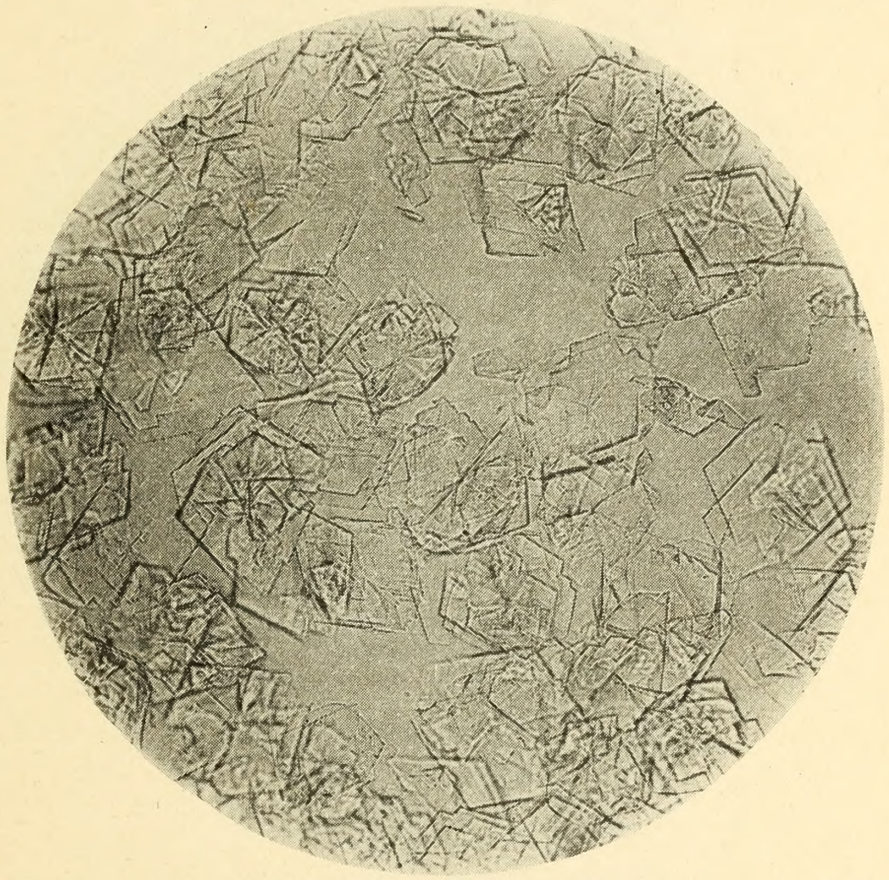


FIG. 15.—Phenylalanine. × 100.

alanine and by tryptophane, the three amino acids having benzene nuclei. However, while tryptophane and tyrosine give the reaction readily, phenylalanine, according to Vickery,* does not respond to the test.

3. *Adamkiewicz reaction.*[60] This reaction is characteristic of tryptophane. If a solution containing tryptophane, either free or combined as in the molecule of most proteins, is added to an equal quantity of a mixture of concentrated sulfuric acid and glacial acetic acid (1:2) a reddish-violet color is obtained, slowly at ordinary temperatures, but more quickly on heating. The reaction is more delicate if the solution

* Vickery, H. B., private communication.

[60] Adamkiewicz, A., *Arch. ges. Physiol.*, 1874, ix, 156; *Ber. chem. Ges.*, 1875, viii, 161.

to be tested is mixed with an equal quantity of glacial acetic acid and the mixture floated on top of an equal volume of concentrated sulfuric acid. At the zone of contact a violet ring forms if the solution contains tryptophane.

This reaction depends upon the presence of aldehydes in the acetic acid. Hopkins and Cole [61] found that most samples of glacial acetic acid which have stood some time contain some glyoxylic acid, HCO.COOH, and it is this aldehyde-acid which is important in the reaction. The test

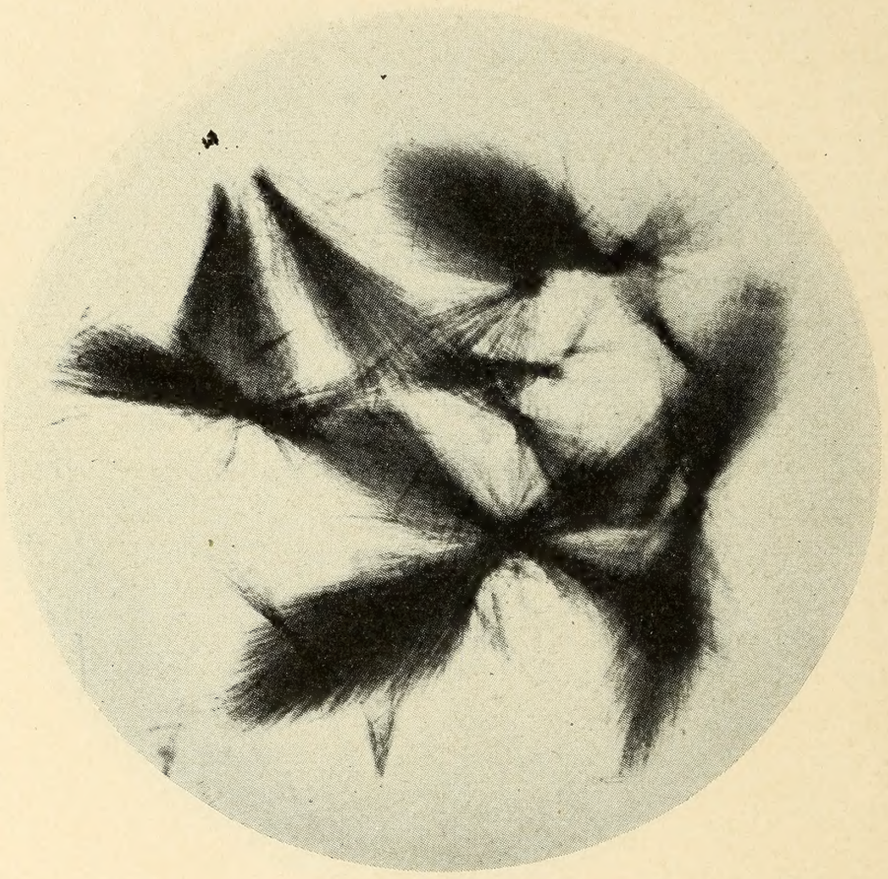


FIG. 16.—Tyrosine. × 120.

may be performed, therefore, using glyoxylic acid instead of acetic acid. Glyoxylic acid is easily made by reducing oxalic acid with powdered magnesium or sodium amalgam.

Glyoxylic acid is known as Hopkins-Cole reagent and when the test is carried out using this acid the reaction is known as the Hopkins-Cole reaction or as the Adamkiewicz-Hopkins test. The mechanism of the reaction is unknown.

Benedict [62] has suggested the use of the magnesium salt of glyoxylic acid as a reagent in carrying out the Hopkins-Cole test.

[61] Hopkins, F. G., and Cole, S. W., *Proc. Roy. Soc.* (*London*), 1901, lxviii, 23.
[62] Benedict, S. R., *J. Biol. Chem.*, 1909, vi, 51.

According to Komm [63] the tryptophane-aldehyde reaction takes place much more slowly with free tryptophane than when tryptophane is a part of the protein molecule or when free tryptophane is in the presence of a suitable accelerator. Substances most effective in hastening the reaction are prolines or protein products containing proline and proline derivatives.

4. *Liebermann's reaction.* Another color reaction involving tryptophane is that of Liebermann,[64] who found that protein when treated

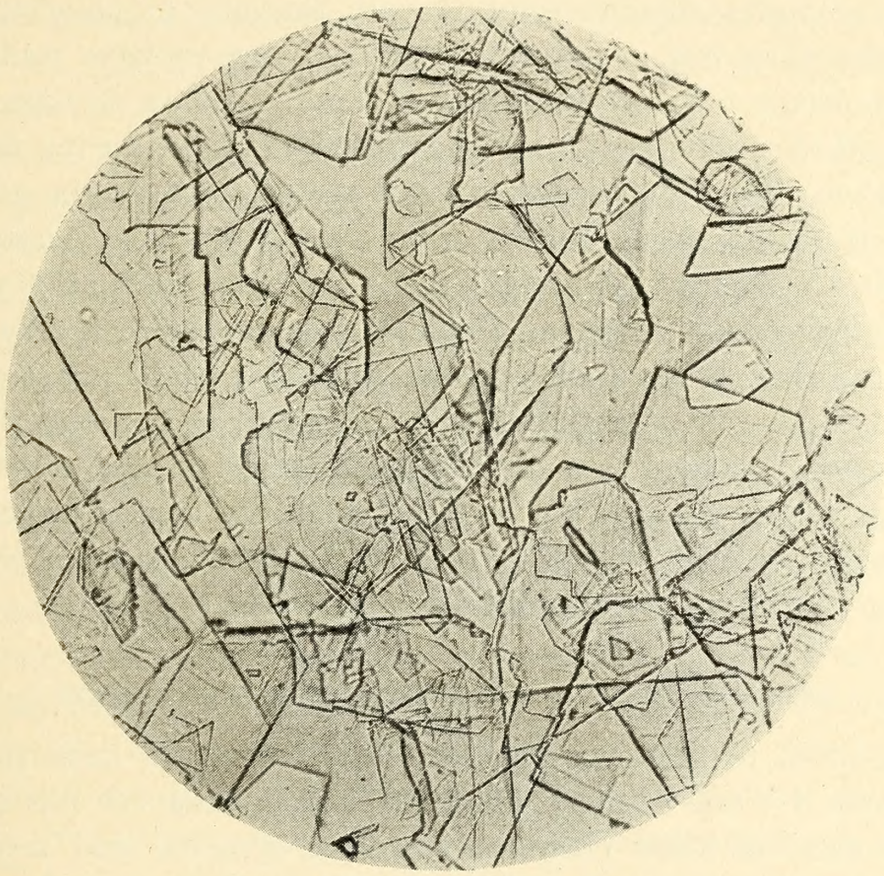


FIG. 17.—Tryptophane. × 100.

first with alcohol and ether and then with hydrochloric acid will develop a violet or bright blue color. This color is due, according to Cole,[65] to a contamination of the ether with glyoxylic acid, which reacts with the tryptophane split off by the hydrochloric acid.

5. *Ninhydrin reaction.*[66] A very sensitive reagent for most amino acids and for some other substances as well is Abderhalden and Schmidt's [67] triketo-hydrindene hydrate (also called ninhydrin),

$$C_6H_4<\begin{matrix}CO\\ \\ CO\end{matrix}>C.(OH)_2.$$

[63] Komm, E., *Z. physiol. Chem.*, 1924, cxl, 74.
[64] Liebermann, *Centr. med. Wissensch.*, 1887.
[65] Cole, S. W., *J. Physiol.*, 1903, xxx, 311.
[66] Ruhemann, S., *J. Chem. Soc., Trans.*, 1910, xcvii, 2025. *See also* Retinger, J. M., *J. Am. Chem. Soc.*, 1917, xxxix, 1059.
[67] Abderhalden, E., and Schmidt, H., *Z. physiol. Chem.*, 1911, lxxii, 37.

A blue color develops on boiling. The test is given by amino acids which have at least one free carboxyl and a free amino group. It is therefore positive with all the amino acids with the exception of proline and hydroxyproline. Histidine after a while develops a Burgundy red color. Glycine will give the reaction in a 1 : 10,000 solution. By means of this valuable reagent it is possible to show the presence of amino acids in fresh urine and in protein-free blood serum.

6. *Tryptophane or Bromine reaction.*[68] Tryptophane when free gives in a faintly alkaline solution with bromine or chlorine water a violet color. The test is not given with the tryptophane united in the protein molecule and may, therefore, be used to follow the course of the splitting off of tryptophane from the protein molecule during the process of digestion. The color developed from tryptophane by the addition of bromine is readily distinguished from the color due to histidine. The former develops in the cold and is easily extracted with amyl alcohol.

7. *Folin's test.* Folin and Denis [69] have developed a very sensitive test for tyrosine. When their reagent, consisting of 10 per cent sodium tungstate, 2 per cent phosphomolybdic acid, and 10 per cent phosphoric acid in water, is added to a solution containing tyrosine, either free or combined, a blue color is developed even in the cold. The test can be made sensitive with 1 part of tyrosine in 1,000,000 parts of water. The reagent acts with all oxybenzol compounds.

SPECIFIC COLOR REACTIONS OF THE AMINO ACIDS

In addition to these widely used tests there is a large number of tests specific for one or more amino acids and which are less commonly used. Because of their chemical structure tyrosine and tryptophane, especially, lend themselves to the formation of colored derivatives.

Tyrosine. Millon's, Folin's, and the xantho-proteic reactions, the first two being specific for tyrosine while the third is specific for tyrosine and tryptophane, have been discussed. Piria's [70] test for tyrosine consists in the development of a violet color when ferric chloride is added to a solution resulting when tyrosine is dissolved in concentrated sulfuric acid, cooled, diluted, neutralized with barium carbonate and filtered. Denigès' [71] test for tyrosine, as modified by Mörner,[72] consists in the development of a permanent green color when tyrosine, either solid or in solution, is boiled with a few cc. of a formalin-sulfuric acid solution (1 vol.

[68] Tiedemann and Gruelin, "Die Verdauung nach Versuchen, Heidelberg and Leipzig," 1826; C. Bernard, *Compt. rend. Acad.*, Suppl. 1855, i.
[69] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xii, 239.
[70] Piria, A., *Ann. Chem.*, 1852, lxxxii, 251.
[71] Denigès, G., *Compt. rend. Acad.*, 1900, cxxx, 583.
[72] Mörner, C. T., *Z. physiol. Chem.*, 1902, xxxvii, 86.

formalin, 45 vol. water and 55 vol. concentrated sulfuric acid). Pauly's [73] test for tyrosine consists in the development of a cherry red color when a solution containing tyrosine is made alkaline with sodium carbonate and then treated with diazo-benzene-sulfonic acid. This coloration may be distinguished from a similar color developed by histidine under like treatment by the fact that the histidine color is a little deeper red which takes on a yellowish tint when diluted.

Biological tests for tyrosine have also been developed. If an aqueous solution of tyrosine is decomposed by certain fungi, for example *Russula delica,* a red color is developed. The formation of this coloring matter can be traced to the action of tyrosinase.[74, 75, 76]

Wurster's test [77] consists in the development of a red color when a boiling aqueous solution of tyrosine is treated with one per cent acetic acid and then dropwise with a solution of one per cent sodium nitrite. The addition of a little dry quinone causes the solution to become a ruby red. Finally, according to Aloy and Rabaut,[78] an aqueous suspension or a hydrochloric acid solution of tyrosine gives with an excess of chlorine water and ammonia a beautiful red color. The delicacy of the reaction is increased if the solution, after the addition of the chlorine water, is shaken with ether, then a drop of ammonia added at the zone of contact of the two liquids. The color developed at the zone of contact is readily distinguished even in very dilute solutions of tyrosine.

Tryptophane. More color reactions have been noted with tryptophane than with any other amino acid. The ability of tryptophane to form colored derivatives is readily explained by its derivation from indole, which stands in close relationship to the important color groups of indigo. It may be noted that of the seven most commonly used tests for the presence of amino acids, three (Adamkiewicz or Hopkins, Liebermann, and the bromine reaction) are specific for tryptophane, and one, the xantho-proteic, is positive with tryptophane and tyrosine. The ninhydrin test is also positive with tryptophane.

The basis of a large number of reactions is the formation of colored compounds when tryptophane is treated with an aldehyde. While the use of various aldehydes [79, 80, 81, 82, 83] has been suggested, the most important tests are those using formaldehyde (Rosenheim), glyoxylic acid

[73] Pauly, H., *Z. physiol. Chem.,* 1904, xlii, 508.
[74] Bertrand, G., *Compt. rend Acad.,* 1896, cxxii, 1215; 1896, cxxiii, 463; 1907, cxlv, 1352.
[75] Chodat, R., *Arch. sci. phys. et nat.,* 1907, xxiv (4), 172.
[76] Abderhalden, E., and Guggenheim, M., *Z. physiol. Chem.,* 1908, liv, 331.
[77] Wurster, C., *Centr. Physiol.,* 1888, i, 193.
[78] Aloy and Rabaut, Ch., *Bull. soc. chim.,* 1908 [4], iii, 391.
[79] Rohde, E., *Z. physiol. Chem.,* 1905, xliv, 161.
[80] Sasaki, T., *Biochem. Z.,* 1910, xxiii, 402.
[81] Dakin, H. D., *J. Biol. Chem.,* 1907, ii, 289.
[82] Granström, E., *Beitr. chem. Physiol. Pathol.,* 1908, xi, 132.
[83] Heimrod, G. W., and Levene, P. A., *Biochem. Z.,* 1910, xxv, 18.

(Adamkiewicz, Adamkiewicz-Hopkins, and Hopkins-Cole), and benzaldehyde (Cole). The Adamkiewicz reaction with its modifications has been mentioned.

In 1903 Cole [65] drew attention to the clear blue color produced when a tryptophane solution was added to a benzaldehyde-sulfuric acid solution. The tryptophane benzaldehyde blue is one of the more stable pigments and seems to be well suited for colorimetric work as well as being a good reagent for quantitative work. Rosenheim [84] showed that the Adamkiewicz color for tryptophane was given by a formaldehyde-sulfuric acid solution. He suggests a dilution of 1 : 2500 formaldehyde solution and the use of pure sulfuric acid to which has been added a small quantity of ferric chloride (5 mg. to 100 cc.) or potassium nitrite (2 mg. to 100 cc.). If the sulfuric acid reagent is run beneath the tryptophane solution a purple ring soon develops at the surface of contact.

Mottram [85] has shown that the Hopkins-Cole test gives two distinct colors with tryptophane according to the amount of aldehyde used. Fearon [86] confirmed this and showed that the same is true in the Rosenheim test. With the minimum amount of aldehyde and the test-tube kept cool, the color in each case is carmine. Using more aldehyde and allowing the temperature to rise, the color is violet to blue. Fearon also showed that the Hopkins-Cole test is due to glyoxylic acid and that the Rosenheim test is due to formaldehyde, a matter over which there has been considerable controversy. Fearon suggests a simple quantitative reagent for tryptophane and indoles. The reagent consists of 10 per cent salicyl aldehyde in alcohol which must be free from acetone. A small quantity of the liquid under examination is mixed with an excess of strong hydrochloric acid. A few drops of the reagent are added and the mixture warmed. A drop of 10 per cent hydrogen peroxide is then added and the warming continued. An intense blue denotes tryptophane, a deep purple scatole, and a bright carmine indole.

According to Ehrlich,[87] *p*-dimethyl-amino-benzaldehyde dissolved in concentrated sulfuric acid and run beneath a solution of protein produces a red-violet ring at the zone of junction. The reaction depends, according to Rohde,[79] upon the presence of tryptophane in the molecule.

Voisenet's [88] reaction for tryptophane consists in the development of a violet color when an aqueous solution or suspension of protein is treated with concentrated hydrochloric acid containing a trace of nitrite in the presence of a drop of formaldehyde.

[84] Rosenheim, O., *Biochem. J.*, 1906, i, 233.
[85] Mottram, V. H., *Biochem J.*, 1913, vii, 249.
[86] Fearon, W. R., *Biochem. J.*, 1920, xiv, 548.
[87] Ehrlich, F., *Med. Woche*, 1901, April.
[88] Voisenet, E., *Bull. soc. chim.*, 1905 [3], xxxiii, 1198.

A biological test for tryptophane has also been suggested. A series of investigations by Cornish and Williams,[89] Venn,[90] and Mattick and Williams [91] has shown that a yellow to orange color is developed when certain members of gram-negative organisms, for example *B. proteus vulgaris,* are allowed to grow in an alkaline medium containing tryptophane. This does not seem to be specific for tryptophane, however, since media containing histidine, alanine, and tyrosine also become similarly colored. In H. Zipfel's method for tryptophane, which depends upon the liberation of indole (and its subsequent detection) from media containing tryptophane by certain indole-forming bacteria, it is necessary, according to Barthel,[92] to neutralize the solution in order to insure growth of the indole-forming bacteria.

Danila [93] has shown that an aqueous solution of free tryptophane, warmed to boiling with a solution of iodic acid, has the power of setting free the iodine.

Romieu [94] has recently described a not altogether characteristic test for tryptophane. When sirupy phosphoric acid is added to protein material and the mixture gently heated a garnet-red color turning to purple is developed. Blanchetière,[95] however, states that with pure tryptophane, Romieu's reaction produces a pale yellow color with a faint green fluorescence instead of the garnet-red changing to purple color. Romieu's reaction is characteristic of substances having an aldehydic function. The blue or violet colorations shown by tryptophane in the protein molecule are not given by glycine, alanine, valine, leucine, phenylalanine, tyrosine, aspartic acid, glutamic acid, serine, proline, cystine or histidine, according to Blanchetière.

Finally there is the pyrrole reaction for tryptophane. If a pine stick previously moistened with hydrochloric acid and washed with water is introduced into a concentrated tryptophane solution, it becomes purple on drying.

Cystine. Cystine is usually detected by decomposing it by boiling with an alkali forming the alkali sulfide, which can be detected by lead acetate or sodium nitroprusside. The former or lead-sulfur [25] test is carried out by boiling the material to be tested with alkali and a lead salt such as lead acetate. The formation of a gray precipitate which gradually changes to a grayish-black is indicative of cystine. Arnold's [96]

[89] Cornish, E. C. V., and Williams, R. S., *Biochem. J.,* 1917, xi, 180.
[90] Venn, E. C. V., *Biochem. J.,* 1920, xiv, 99.
[91] Mattick, E. C. V., and Williams, R. S., *Biochem. J.,* 1921, xv, 213.
[92] Barthel, C., *J. Bact.* 1921, vi, 85.
[93] Danila, P., *Compt. rend. soc. biol.,* 1923, lxxxviii, 278.
[94] Romieu, M., *Compt. rend. Acad.,* 1925, clxxx, 875.
[95] Blanchetière, A., *Compt. rend. Acad.,* 1925, clxxx, 2072.
[96] Arnold, V., *Z. physiol. Chem.,* 1911, lxx, 300.

nitroprusside test for cystine, as modified by Hopkins,[97] consists in adding to one or two cc. of the solution to be tested an excess of finely powdered ammonium sulfate, followed by two to four drops of fresh sodium nitroprusside (5 per cent); on making alkaline with ammonia, an intense purple color develops.

Sullivan's [98] test for cystine depends upon the fact that of many amino acids and sulfur-containing compounds, cysteine is the only one that gives a red color with sodium-β-naphtho-quinone-4-sulfonate in the presence of alkali and a reducing agent such as sodium sulfite. Cystine gives this test only very slowly, but if sodium cyanide is added to a cystine solution before the addition of the naphthoquinone and sulfite, the characteristic color for cysteine is obtained, because the cyanide reduces the cystine to cysteine.

Glycine. An aqueous solution of glycine becomes deep red on the addition of ferric chloride. This color disappears on the addition of acid and appears again on decomposition with ammonia.[99] If a drop of phenol is added to a glycine solution and then sodium hypochlorite, a blue color is obtained.[100]

Leucine. A trace of crystalline quinone added to a boiling solution of leucine produces a red color; this is especially prompt if solid sodium chloride is added. A very sensitive violet coloration is produced when to a cold solution of leucine a very small amount of solid quinone and a drop of sodium carbonate solution are added.[101]

Phenylalanine. This amino acid is converted into the hydroxy acid by means of nitrous acid. When a small amount of phenyllactic acid is decomposed by heat, phenyllactaldehyde is formed. By adding 2 to 3 cc. of a 50 per cent alcoholic sulfuric acid solution to this aldehyde, a permanent green color develops after a few hours or overnight.[102] This may serve as a much better qualitative test for phenylalanine than Fischer's method which depends on detecting the odor of phenylacetaldehyde after the oxidation of phenylalanine with dichromate and sulfuric acid.

Histidine—Knoop's Test.[103] If, to a slightly acid solution containing histidine, bromine water is added in excess and then the excess bromine boiled off, a brownish red color will be developed. The only other substance found to give this test is histamine. The test is given with

[97] Hopkins, F. G., *Biochem. J.*, 1921, xv, 286.
[98] Sullivan, M. X., *Abst. Bact.*, 1925, ix, 37.
[99] Engel, E., *Z. anal. Chem.*, 1876, xv, 344.
[100] Engel, E., *Ber. cnem. Ges.*, 1875, viii, 699.
[101] Wurster, C., *Centr. Physiol.*, 1888, ii, 590.
[102] Kodama, S., *J. Tokyo Chem. Soc.*, 1919, xl, 743.
[103] Knoop, F., *Beitr. chem. Physiol. Pathol.*, 1908, xi, 356.

histidine in a dilution of 1 : 1000. Hunter [104] has increased the delicacy of this reaction and rendered it more certain in colored solutions by using the correct amounts of bromine. The color developed from tryptophane by the addition of bromine is readily distinguished from the color due to histidine. The former develops in the cold and may be easily extracted with amyl alcohol. The colored substance in Knoop's test is not extracted by any of the ordinary solvents.

Pauly's [73] diazo reactions of histidine and tyrosine have been made specific for histidine by Inouye [105] and by Totani.[106] Inouye noticed that when tyrosine and histidine were submitted to the Schotten-Baumann reaction, the former was converted into its dibenzoyl derivative and loses its property of coupling with diazotised sulfanilic acid, while the latter, though forming its monobenzoyl derivative, still maintained unaltered its ability of yielding a red coloration in the presence of the diazonium acid. He therefore suggests treatment of the solution to be tested first with benzoyl chloride and sodium carbonate in order to prevent the tyrosine from combining with the diazo acid in the subsequent diazotisation. In order to apply Inouye's test for histidine it is first necessary to hydrolyze the protein containing it.

Totani made Pauly's diazo reaction specific for histidine by reducing the similarly colored diazo compounds with zinc dust in hydrochloric acid solution, subsequently rendering the solution alkaline with an excess of ammonia. Under these conditions tyrosine gives a rose-red and histidine a golden yellow coloration. While this modification is specific for histidine and may be used for histidine either free or combined, it is not specific for tyrosine, since cystine, alanine, phenylalanine, asparagine and some other organic compounds also give a reddish coloration. The reddish colorations produced by tyrosine and other amino acids on reduction of the diazo compounds are readily destroyed by hydrogen peroxide, while the golden yellow color of histidine is little altered.

Arginine. According to Sakaguchi [107] when to 3 cc. of a protein solution made alkaline with NaOH are added 2 drops of a 0.1 per cent α-naphthol in 70 per cent alcohol solution, then several drops of a 5 per cent NaOCl solution, a beautiful red color develops in a short time. The sensitivity of this reaction is 1 : 50,000 of protein and 1 : 1,000,000 of arginine. Of the protein cleavage products only arginine gives this reaction. The reaction is due to the guanidine grouping in arginine.

[104] Hunter, G., *Biochem. J.*, 1922, xvi, 637.
[105] Inouye, K., *Z. physiol. Chem.*, 1913, lxxxiii, 79.
[106] Totani, G., *Biochem. J.*, 1915, ix, 385.
[107] Sakaguchi, S., *J. Biochem. (Japan)*, 1925, v, 25.

PRECIPITANTS FOR THE AMINO ACIDS

The amino acids are, in general, precipitated by the so-called alkaloidal reagents, picric acid, picrolonic acid, phosphotungstic acid, mercuric chloride, gold chloride, platinum chloride, iodopotassium iodide, potassium-cadmium iodide, potassium-bismuthous iodide, potassium-mercuric chloride, potassium-zinc iodide, phospho-molybdic acid, and tannic acid. Mercuric nitrate, silver nitrate, cupric chloride, and mercuric acetate are also often used for this purpose.

The neutral salts of many metals form double salts of definite composition with the amino acids and in a large number of cases these double salts are crystalline and may be used for identification purposes. The silver, copper, mercury, lead, cadmium, zinc, calcium and barium salts are commonly used as derivatives of the amino acids for identification. The reaction with cupric chloride may be written as follows:

$$\begin{array}{l} \mathrm{R.CH.COOH} \\ \quad\vert \\ \quad\mathrm{NH_2} \end{array} + \mathrm{CuCl_2} = \begin{array}{l} \mathrm{R.CH.COOH} \\ \quad\vert \\ \quad\mathrm{NH_2.CuCl_2} \end{array}$$

Probably one of the best precipitants for the amino acids is mercuric acetate in the presence of carbonates.[108] The reaction is also probably quantitative.

Cupric oxide or hydroxide, under certain conditions, will form crystalline complexes with the amino acids. These have been used in various ways, especially for the identification of the amino acids. Kober and Sugiura[109] have developed a procedure using cupric hydroxide whereby the formation of the copper complexes of amino acids (also peptides and peptones) in neutral or slightly alkaline solution has been improved so that quantitative results can be obtained in very dilute solution (one part in 500,000). It can therefore be used both as a valuable qualitative as well as a quantitative test for the amino acids.

Among the compounds capable of precipitating the amino acids are certain organic compounds. For precipitating the easily soluble amino acids from their mixtures or solutions containing salts and other foreign bodies, benzoyl, or more often phenylcyanate, derivatives have been used. β-naphthalene-sulfochloride seems to be especially satisfactory.[110] This reagent combines with the amino acids in alkaline solutions and precipitates them.

In this connection it might be pointed out that lead oxide has been frequently used to free mixtures of amino acids from sulfuric and

[108] Neuberg, C., and Kerb, J., *Biochem. Z.*, 1912, xl, 498; 1914, lxvii, 119.
[109] Kober, P. A., and Sugiura, K., *J. Biol. Chem.*, 1912, xiii, 1; *J. Amer. Chem. Soc.*, 1913, xxxv, 1546.
[110] Fischer, E., and Bergell, P., *Ber. chem. Ges.*, 1902, xxxv, 3779.

especially hydrochloric acid.[111] Levene and Van Slyke [112] have pointed out that tyrosine and aspartic acid form nearly insoluble compounds with lead when subjected to thorough boiling with the precipitated oxide for 15 to 20 minutes. From this observation it appears that this procedure, if excess of oxide is used, is likely to remove aspartic acid and tyrosine from such solutions.

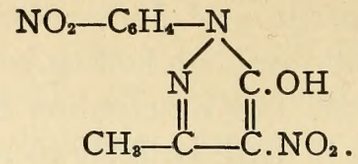
Pfeiffer and his co-workers [113] have made extensive investigations concerning the addition products with neutral salts. They have studied particularly the addition products of the halides of the alkali and alkaline earth metals with glycine, its simpler derivatives, and alanine. They have obtained definite compounds of glycine with barium chloride, barium bromide, strontium chloride and bromide, calcium chloride and bromide, and with magnesium and lithium chlorides. They obtained no definite compounds with potassium nitrate, potassium chloride or potassium bromide. The crystalline addition complexes contain the components in some simple molecular proportion. Thus, calcium chloride was found to furnish three compounds, $CaCl_2.C_2H_5O_2N.3H_2O$; $CaCl_2.(C_2H_5O_2N)_2,4H_2O$; and $CaCl_2.(C_2H_5O)_3$; and lithium bromide two compounds, $LiBr.C_2H_5O_2N.H_2O$; and $LiBr.(C_2H_5O_2N)_2.H_2O$. These addition compounds are soluble in water and are obtained from an aqueous solution on evaporation or on the addition of alcohol. These investigators have also pointed out that tyrosine and aspartic acid in addition to glycine are not salted out of their saturated solutions by saturating with the chlorides, bromides, and sulfates of the alkalies, alkali earths, ammonia, potassium and sodium acetates, $AlCl_3$, $Al_2(SO_4)_3$, and $(CO_2K)_2$.

Bayliss [114] has on several occasions taken exception to the work of Pfeiffer and his associates, for some preliminary experiments of his own have indicated that the problem was more complex and involved the formation of mixed crystals. King and Palmer,[115] however, have repeted much of Pfeiffer's work and have confirmed it to a large extent.

Another salt which deserves mention as a precipitant of certain amino acids is mercuric sulfate. It precipitates histidine and tryptophane quantitatively, and cystine and tyrosine to a less extent. Using this reagent, Onslow [116] has suggested a quantitative method for the determination of tryptophane.

[111] Abderhalden, E., "Handbuch Biochem. Arbeitsmethoden," 1910 (Berlin), ii, 482.
[112] Levene, P. A., and Van Slyke, D. D., *J. Biol. Chem.*, 1910, viii, 285.
[113] Pfeiffer, P., and Modelski, J. v., *Z. physiol. Chem.*, 1912, lxxxi, 329; 1913, lxxxv, 1; Pfeiffer, P., and Wittka, Fr., *Ber. chem. Ges.*, 1915, xlviii, 1041; 1915, xlviii, 1289; Pfeiffer, P., Würgler, J., and Wittka, Fr., *Ber. chem. Ges.*, 1915, xlviii, 1938; Pfeiffer, P., and Würgler, J., *Z. physiol. Chem.*, 1916, xcvii, 128.
[114] Bayliss, W. M., "Principles of General Physiology," 1915 Edition, 105, 220, 281; Bayliss, W. M., *J. Physiol.*, 1919, liii, 162.
[115] King, H., and Palmer, A. D., *Biochem. J.*, 1920, xiv, 574.
[116] Onslow, H., *Biochem. J.*, 1921, xv, 392; 1924, xviii, 63.

An organic acid which deserves special mention is picrolonic acid,

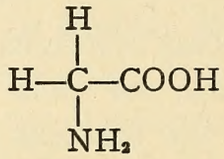


This acid was introduced by Knorr [117] as a precipitant for organic bases, and Steudel [118] has applied it to the hexone bases obtained by the hydrolysis of proteins. Mayeda [119] has described the picrolonates of the aromatic amino acids, tryptophane and phenylalanine. Abderhalden and Weil [120] have described the picrolonates of alanine, glycine, and inactive leucine. Levene and Van Slyke,[121] in a rather extensive investigation of the picrolonates of the amino acids, found that all the natural monoamino acids, with the exception of proline and oxyproline, gave crystalline, definite salts with picrolonic acid, most of them being fairly insoluble in cold water. The picrolonate of phenylalanine is, in particular, noted for its insolubility.

In an investigation by Whitehorn [122] it was shown that the strongly basic amino acids, lysine, histidine, and arginine, were completely removed from solution by filtration through the zeolite "permutit." The monoamino-monocarboxylic acids were not absorbed. A saturated solution of potassium chloride is recommended for use in the recovery of the bases taken up by permutit.

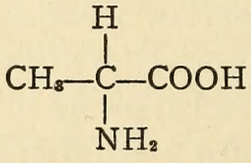
OPTICAL PROPERTIES

From an examination of the structural formula of glycine it is evident that only one isomer of this substance is possible.



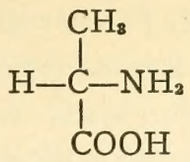
Glycine

In alanine, however, the α-carbon atom

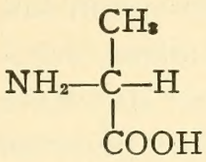


is attached to four *different* groups. In other words, alanine contains an asymmetric carbon atom and, therefore, the possibility of stereoisomerism. If the compound

[117] Knorr, L., *Ber. chem. Ges.*, 1897, xxx, 909.
[118] Steudel, H., *Z. physiol. Chem.*, 1903, xxxvii, 219.
[119] Mayeda, M., *Z. physiol. Chem.*, 1907, li, 261.
[120] Abderhalden, E., and Weil, A., *Z. physiol. Chem.*, 1912, lxxviii, 150.
[121] Levene, P. A., and Van Slyke, D. D., *J. Biol. Chem.*, 1912, xii, 127.
[122] Whitehorn, J. C., *J. Biol. Chem.*, 1923, lvi, 751.



rotates the plane of polarized light to the right, then its stereoisomer



will rotate the plane of polarized light to the left, and it will be possible to obtain a racemic modification without any influence on polarized light by mixing an equal number of molecules of the two isomeric forms.

It is probable that only one of the active forms of any amino acid occurs naturally in the protein molecule. The majority of the amino acids obtained from proteins by acid hydrolysis or by digestive enzymes are laevo-rotatory, although almost as many are dextro-rotatory. The dextro-rotatory acids are designated *d*-acids, while the laevo-rotatory acids are designated *l*-acids. The optically active alanine which is found as a normal constituent of the protein molecule is properly designated *d*-alanine because it is dextro-rotatory.

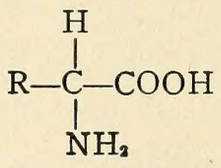
All the amino acids so far obtained from proteins by acid hydrolysis or by digestive enzymes are all optically active, with the exception of glycine. Those amino acids obtained by synthetic methods are inactive and special methods must be used in order to obtain from the artificially formed racemic mixture the active acid desired. The separation from the racemic modifications or mixtures of the component active compounds is called "resolution." A number of different methods may be used to resolve a racemic mixture into its active components. If, in case of a racemic compound, the proper conditions during crystallization are maintained, a mixture of enantiomorphic crystals which may be separated mechanically often forms. Such was the observation of Pasteur in the case of sodium-ammonium racemate. Pasteur is likewise responsible for two other methods of resolution in common use. Racemic acids, such as the synthetic amino acids, are combined with an optically active base, *e.g.,* an alkaloid. In the case of racemic acid, Pasteur used *l*-cinchonine. The two salts formed are (a) *d*-acid + *l*-base, and (b) *l*-acid + *l*-base. Since these two salts are not enantiomorphously related, they possess different physical properties, such as solubilities, and may therefore be separated by fractional crystallization.

The ordinary α-amino acids are so feebly acidic that their resolution into optically active components by means of their salts formed with

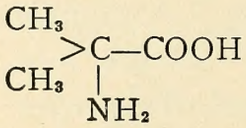
active bases cannot be effected. Fischer and his co-workers have shown, however, that the acyl derivatives, especially the formyl, acetyl and benzoyl compounds of the amino acids, form well crystallized salts with alkaloids, from which active amino acids may be recovered by hydrolysis.

The third method of resolution originated by Pasteur consists in subjecting a solution of an ammonium salt of the acid to the action of some of the lower plant organisms, *e.g.,* molds, bacteria, yeasts, etc. Different organisms are required in different cases. Pasteur found that ordinary green mold—*Penicillium glaucum*—when grown in a solution of ammonium racemate, destroys the salt of the *d*-acid and leaves a solution of the salt of the *l*-acid. If, however, the decomposition is allowed to proceed, the *l*-salt is also destroyed; the reaction is a preferential decomposition, and, if stopped at a suitable time, practically all *d*-salt will have disappeared. It is obvious that in this method one of the active components is lost; but by using two distinct organisms in separate solutions it is sometimes possible to obtain both *d*- and *l*-compounds. Ehrlich has used this method on a large scale in the preparation of active amino acids.

The type formula of the amino acids is

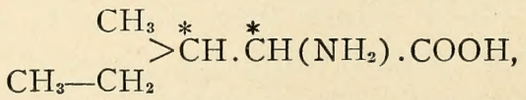


in which the α-carbon atom is, in all cases except glycine, connected with four different groups or atoms. It is the α-carbon atom, therefore, that is asymmetric. However, if α-amino-iso-butyric acid,



an acid claimed by Foreman [24] and also by Abderhalden and Weil [35] and still more recently by Abderhalden [36] to be present in proteins, is proven to be a normal constituent of the protein molecule, it will be of especial interest because it will be the second optically inactive amino acid and the first to contain a tertiary carbon atom to be found in proteins.

Iso-leucine,

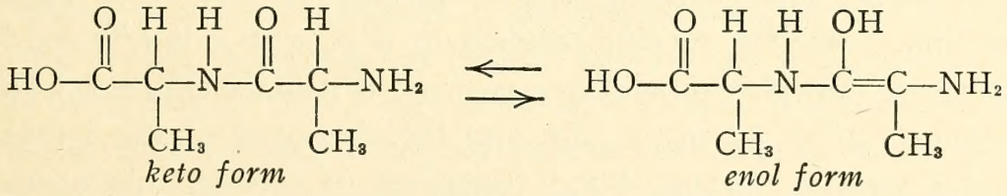


contains two asymmetric carbon atoms and may, therefore, exist in four optically active forms, *i.e.,* two isomeric *d*-isoleucines and two *l*-iso-

leucines. Since *d*-isoleucine is the form occurring in proteins, the two *d*-isoleucines only are of biological interest. When the *d*-isoleucine normally found in proteins is heated for 20 hours in an autoclave at 180° with barium hydroxide the solution gradually becomes levo-rotatory, which rotation soon becomes constant. The solution contains equal amounts of *d*-isoleucine and an isomer, called d'allo-isoleucine, produced by the rearrangement of the groups attached to one of the two carbon atoms. Both forms of *d*-isoleucine may be synthesized.[123, 124, 125, 126, 127]

While the amino acids obtained from proteins by acid hydrolysis are all, with the exception of glycine, optically active, those obtained by hydrolysis with alkalies, or which have been obtained by acid hydrolysis from proteins which have been treated for a time with dilute alkali, are for the most part inactive. The amino acids obtained from such alkali-treated proteins are as a rule, but not exclusively, composed of equal amounts of the dextro- and laevo-rotatory forms of the acids. It follows, therefore, that by the action of the alkali, the optically active form of the acid normally occurring in the protein molecule is changed into its optically active isomer. This process by which from one optically active isomer the opposite optically active isomer is produced is called "racemization." The amino acids when free, however, are not easily or rapidly racemized by dilute alkali. The racemization produced by the action of dilute alkali on proteins must, therefore, occur while the amino acids are combined in the molecule of the protein. The way this is produced is explained by Dakin [128, 129] as follows:

"While salts of the free amino acids cannot exhibit keto-enol isomerism, it is possible for certain groups of the peptide complex to exhibit this type of isomerism. Alanyl-alanine, for example, under the influence of dilute alkali at low temperatures may, by intramolecular rearrangement of the hydrogen atom attached to α-carbon atom, pass over to the oxygen atom of the adjacent ketone group, thus



keto form *enol form*

The keto and enol forms are probably in equilibrium, some keto going to the enol form and some enol to the keto form. While in the

[123] Bouveault, L., and Locquin, R., *Compt. rend. Acad.*, 1905, cxli, 115.
[124] Locquin, R., *Bull. soc. chim.*, 1907 (4), i, 595, 601.
[125] Ehrlich, F., *Ber. chem. Ges.*, 1908, xli, 1453.
[126] Brasch, W., and Friedmann, E., *Beitr. chem. Physiol. Pathol.*, 1908, xi, 376.
[127] Abderhalden, E., Hirsch. P., and Schuler, J., *Ber. chem. Ges.*, 1909, xlii, 3394.
[128] Dakin, H. D., *J. Biol. Chem.*, 1912, xiii, 357.
[129] Dakin, H. D., and Dudley, H. W., *J. Biol. Chem.*, 1913, xv, 263.

keto form the α-carbon atom of the second alanyl group is asymmetric, in the enol form it becomes symmetrical. When the enol form changes back to the keto form with its asymmetrical carbon atom equal amounts of the two optical isomers will be formed, since there is nothing to determine that one isomer rather than the other shall be formed. The alkali must assist in producing the enol form in the peptide or larger molecular complex and thus cause the racemization. Of the two amino acid groups present in the above peptide complex only the one containing the —CO—CH— group could exhibit keto-enol tautomerism and hence racemization; the terminal amino acid group containing a free carboxyl group would be unchanged.

Since only those amino acids which have their carboxyl groups combined can undergo this enol formation, it is possible to find out, by studying the optical activity of various amino acids produced from proteins by acid hydrolysis after the protein has been treated with dilute alkali, which of the acids occupied terminal positions in the molecule and which did not. Those which were racemized must have had their carboxyl groups bound; those which were not racemized probably had their carboxyl groups free. By this method Dakin [128] concluded that in gelatin none of the carboxyl groups of leucine, aspartic acid, arginine, histidine or phenylalanine are free, while in glutamic acid, lysine, and probably alanine the carboxyl groups are free or, in other words, according to Dakin, may occupy terminal positions in the peptide chains.

Optically active substances when dissolved or in the liquid state have the power of rotating a plane of polarized light to the right or to the left. The specific rotatory power of a substance, $[\alpha]_D$, is the angle of rotation which the plane of polarization of the *D* line of the spectrum (sodium) would undergo in passing through one decimeter of a solution containing one gram of active substance to one cubic centimeter of solution. Since the specific rotation of a substance is affected by the temperature, that also should be given and is usually expressed $[\alpha]_D^{20°}$. The concentration of the solute, and the character of the solvent also affect the specific rotation. Small quantities of foreign substances, such as inorganic salts, greatly affect the specific rotation also. These facts together with the rather difficult solubility in water of some of the amino acids probably explain the lack of concordant results found in the literature on the specific rotations of the amino acids.

In Table 4 there is summarized the specific rotations of practically all the amino acids in water, acid and alkalies. No attempt has been

made to make this all inclusive, since there is a very large number of determinations on some of the amino acids at least.

The effect of the character of the solvent and the concentration of active substance is shown very clearly by Wood,[130] who determined the optical activity of leucine, aspartic acid and glutamic acid in water and in various concentrations of hydrochloric acid and of sodium hydroxide, and also by Clough,* who studied the influence of hydrochloric acid, sodium hydroxide and some inorganic salts upon the rotation of aspartic acid at different temperatures.

According to Wood the specific rotation of leucine in distilled water is $[\alpha]_D^{25} = -9.84°$. In an acid solution containing 0.55 mol. of HCl to 1 mol. of leucine $[\alpha]_D^{25} = 1.70°$. This value rapidly increases with the increased ratio of HCl to leucine until a ratio of 1 : 1.34 is used when $[\alpha]_D^{25} = +11.20°$. With increased amounts of HCl, however, the specific rotation is little affected. In alkaline solution containing 0.54 mol. of NaOH to one of leucine $[\alpha]_D^{25} = 0.58°$. This value rapidly increases until a ratio of unity is reached when $[\alpha]_D^{25} = 4.00°$. While considerable variation is obtained when increased amounts of alkali are used, still it is quite evident that the effect of alkali on the specific rotation of leucine is of a similar nature to the influence of HCl.

The influence of HCl on the specific rotations of glutamic acid and of aspartic acid is similar to its effect on leucine; *i.e.*, the specific rotation reaches its maximum when the ratio of acid to leucine is slightly above one. In case of glutamic and aspartic acids the effect of alkali is first to decrease the specific rotation. A minimum is reached when the acid salt is formed, corresponding to a ratio of alkali to acid of a little above unity. Then with increased amounts of alkali the specific rotation behaves in a manner similar to that accompanying the increase of acid until a maximum is obtained when the molecular ratio of NaOH to amino acid is a little greater than two, after which there is no appreciable alteration in the magnitude of the specific rotation.

If the specific rotations are plotted against the molecular ratios it will be noted that in every case there is a distinct break in the curve. This break never occurs at a point where the ratio of added acid or base to amino acid is equal to unity, but always at a point showing a preponderance of acid or base; in other words, an excess of acid or base over the amount required to form a salt with the amino acid must be added before this break in the curve is reached. The value of the specific rotation corresponding with the break in the curve may be regarded as the optical activity of the salt of the amino acid.

[130] Wood, J. K., *J. Chem. Soc., Trans.*, 1914, cv, 1988.
* Clough, G. W., *J. Chem. Soc.*, 1915, cvii, 1509.

TABLE 4. *The Specific Rotations of the Amino Acids.*

Amino Acid	Temp. °C.	$[\alpha]_D$	Solution	Ref. No.*
d-Alanine	22	+ 2.7°	Aq.	A1
	20	+10.4°	9-10% HCl	A2
l-Serine	20	— 6.83°	Aq.	A3
	25	+14.45°	*N* HCl	A3
d-Valine	20	+ 6.42°	Aq.	A
	16	+28.2°	20% HCl	A4
	16	+27.9°	20% HCl	A4
	20	+25.9°	20% HCl	A5
	20	+28.7°	20% HCl	A6
l-Leucine	..	—10.35°	Aq.	A7
	25	— 9.84°	Aq.	8
	20	+17.54°	10% HCl	A9
	..	+17.3°	19-15% HCl	A10
	..	+17.3° to 17.76°	20% HCl	A11
	..	+18.9°	24% HCl	A11
	20	+15.7°	20% HCl	A7
	20	+15.59°	21% HCl	A12
	25	+ 1.70° to 11.20°	0.55 M HCl per M to 1.34 M HCl per M	8
	15	+17.75°	20% HCl	13
	24	+ 6.65°	10% KOH	A9
	20	+ 8.05°	4% NaOH	A14
	25	+ 0.58° to 4.00°	0.54 M NaOH per M to 1.0 M NaOH per M	8
d-Isoleucine	20	+ 9.58°	Aq.	15
	20	+11°29′	Aq.	A16
	20	+36.80°	20% HCl	15
	20	+34.26°	20% HCl	A17
	20	+41.29°	20% HCl	A18
	20	+40°61′	20% HCl	A16
	20	+11.1°	dil. alk.	15
d-Norleucine	20	+ 4.5°	Aq.	19
	20	+ 5.16°	Aq.	20
	20	+21°	20% HCl	19
	20	+20.44°	20% HCl	20
l-Aspartic acid	20	+ 4.36°	Aq.	A21
	70	0	Aq.	A21
	90	— 1.86°	Aq.	A21
	25	+ 5.87°	Aq.	8
	14	+ 5.1°	Aq.	22
	25	+ 3.8°	Aq.	22
	35	+ 2.5°	Aq.	22
	48	+ 1.3°	Aq.	22
	20	+25.7°	HCl	A23
	25	+ 9.0° to 23.9°	0.29 M HCl per M to 4.90 M HCl per M	8
	25	+23.4°	HCl (0.2 *N*, 1.5 mols.)	22
	12	+26.2°	HCl (*N*, 1.5 mols.)	22
	20	+25.5°	HCl (*N*, 1.5 mols.)	22
	25	+25.0°	HCl (*N*, 1.5 mols.)	22
	44	+22.9°	HCl (*N*, 1.5 mols.)	22

* NOTE: All references preceded by the letter A are taken from Abderhalden's "Biochemisches Handlexikon," Vol. IV, 1911 (Berlin).

TABLE 4. (*Continued*)

Amino Acid	Temp. °C.	$[\alpha]_D$	Solution	Ref. No.*
l-Aspartic acid (*continued*)	..	+25.16°	HNO_3	A24
	20	— 2.37°	Alk.	A23
	25	— 3.54°	0.42 M NaOH per M	8
	25	—14.7°	0.86 M NaOH per M	8
	25	—14.5°	1.08 M NaOH per M	8
	25	— 9.9°	1.53 M NaOH per M	8
	25	— 5.1°	2.17 M NaOH per M	8
	25	— 3.47°	6.21 M NaOH per M	8
	18	—15.8°	NaOH (0.2 *N*, 1 mol.)	22
	25	—17.3°	NaOH (0.2 *N*, 1 mol.)	22
	46	—20.9°	NaOH (0.2 *N*, 1 mol.)	22
	12	— 3.5°	NaOH (*N*, 1 mol.)	22
	25	— 6.0°	NaOH (*N*, 1 mol.)	22
	25	+ 0.5°	NaOH (2*N*, 1 mol.)	22
	25	— 2.4°	NaOH (*N*, 2 mol.)	22
d-Glutamic acid	21	+10.2° (?)	Aq.	A25
	..	+24.03°	Aq.	A26
	25	+12.62°	Aq.	8
	15	+10.3°	Aq. (p=0.99)	27
	60	+ 6.9°	Aq. (p=0.99)	27
	44	+10.3°	Aq. (p=2.91)	27
	75	+ 7.8°	Aq. (p=2.91)	27
	..	+30.85°	HCl	A28
	20	+31.2°	HCl	19
	25	+23.04° to 32.36°	0.54 M HCl per M 4.53 M HCl per M	8
	25	+13.90° to 24.30°	1.47 M acetic acid per M 4.41 M acetic acid per M	8
	25	+ 3.00°	0.49 M NaOH per M	8
	25	— 0.40°	0.74 M NaOH per M	8
	25	— 3.97°	1.09 M NaOH per M	8
	25	— 0.40°	1.32 M NaOH per M	8
	25	+10.22°	2.47 M NaOH per M	8
	25	+10.90°	5.96 M NaOH per M	8
d-Arginine	For calculated values from salts see Gulewitsch			A29
d-Lysine	20	+14.6°	6.49%, aqueous	65
l-Cystine	..	—223°	HCl	A30
	19.5	—205.9°	11.2% HCl	A31
	..	—214°	dil. HCl	A32
	..	—224.3°	dil. HCl	A33
(hair)	..	—221.9°	.....	A34
("stone")	..	—223.6°	.....	A34
("stone")	..	—218.2°	*N* HCl	A35
(hair)	..	—223.8°	.....	A35
(edestin, hemp)	..	—218.8°	.....	A35
(feathers)	..	—219.8°	.....	A35
(horn)	..	—220.5°	.....	A35
(serum globulin)	..	—221.2°	.....	A35
(serum albumin)	..	—216.8°	.....	A35
(hair)	..	—251.1°	.....	36
("stone")	..	—252.2°	.....	36
(synthetic)	20	—209.6°	*N* HCl	A37
	15	—218°	1%, 20% HCl	13
	20	—242.6°	1%, 0.1 *N* HCl	38
	29	—215°	*N* HCl	39
	..	— 97.5°	NH_4OH	A30
	15	—103°	1%, 1% NH_4OH	13

TABLE 4. (*Continued*)

Amino Acid	Temp. °C.	$[\alpha]_D$	Solution	Ref. No.*
l-Phenylalanine	20	—35.1°	Aq.	A40
	..	—35.3°	Aq.	A41
(L, luteus, albus).....	16	—39.4° Av.	Aq.	A41
(conglutin)	16	—38.1°	Aq.	A42
l-Tyrosine	16	— 7.98°	4%, 21% HCl	A43
	20	— 8.07°	4%, 21% HCl	A44
	..	— 8.48°	4%, 21% HCl	A45
	20	— 8.64°	4%, 21% HCl	A46
	20	—13.2°	4.6%, 4% HCl	A46
	16	—14.6° to 16.1°	.....	A47
	20	—12.9°	9%, 4% HCl	A48
	20	—12.5°	8%, 4% HCl	A48
	20	—16.2°	5%, 4% HCl	A48
	..	—15.6°	4% HCl	A49
	15	— 9.00°	3%, 20% HCl	13
	..	—11.63° Av.	4% HCl	50
	..	— 9.01°	5.8%, 11.6% KOH	A51
l-Tryptophane	..	—33°	Aq.	A52
	..	—29.75° to —40.3°	0.5%, aq.	A53
	..	—30.3°	Aq.	A54
	20	+ 1.31°	6%, *N* HCl	A55
	20	+ 5.7° to 6.3°	2.3%, 0.5 *N* NaOH	A55
	20	+ 6.06° to 6.12°	10-12%, *N* NaOH	A55
	20	+ 6.17° to 6.57°	10%, *N* NaOH	A54
	..	+ 5.56° to 5.69°	2-3,5%, NaOH	A53
	20	+ 5.27°	Alk.	A56
	20	+ 5.27°	Alk.	A54
l-Histidine	..	— 7.59°	Aq.	A57
	26	—38.1°	0.3447 g. in 15 cc. H_2O	58
	20	—39.44°	0.2928 g. in 8.3620 g. aq. sol.	58
	20	—39.65°	0.2161 g. in 9.8168 g. aq. sol.	59
	..	+ 0.18°	1 M HCl per M	A57
	..	+ 1.54°	2 M HCl per M	A57
	..	+ 1.31°	4 M HCl per M	A57
l-Proline	20	—77.40°	7.39%, aq.	A60
	20	—80.9°	6.46%, aq.	A61
	20	—93.0°	2.35%, KOH	A61
	20	—84.9°	Aq.	66
l-Hydroxyproline	20	—81.04°	9.3%, aq.	A62
(synthetic)	20	—75.6°	.....	63
(natural)	20	—80.6°	Aq.	66
	20	—76.3°	.....	63
d-Hydroxyglutamic acid..	..	+ 0.8°	4.0%, aq.	64
	..	+16.3°	2.0%, 20% HCl	64
l-Methionine	20	— 7.2°	2.7%, aq. soln.	67

* NOTE: All references preceded by the letter A are taken from Abderhalden's "Biochem. isches Handlexikon," Vol. IV, 1911 (Berlin).

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TABLE 5. *Optical Features of Amino Acids.**

	Alanine	Aspartic Acid	Cystine	Glutamic Acid	Glycine	Leucine	Phenyl-alanine	Serine	Trypto-phane	Tyro-sine	Valine
Habit	Needles	Plates	Prisms	Plates	Plates	Plates	Plates	Plates	Plates	Needles	Plates
Indices											
$n\alpha$	1.515	1.515	1.640	1.490	1.495	1.525	1.600	1.515	1.625	1.550	1.495
$n\beta$	1.540	1.560	1.700	1.605	1.615	1.535	1.610	1.575	(?)	1.600	(?)
$n\gamma$	1.575	1.630	1.700	1.620	1.650	1.560	1.675	1.586	1.635	1.680	1.565
$n\gamma$-$n\alpha$	0.060	0.115	0.060	0.130	0.155	0.035	0.075	0.071	0.010	0.130	0.070
Usual	1.575	1.630	1.700	1.605	1.615	1.535	1.675	1.576	1.635	1.680	1.565
Colors	1-2	3-4		3-4	1-2	1	1-2	2-3	1	1	1
Extinction	Parallel	(?)	Indeter-minate	(?)	(?)	Parallel (?)	Inclined	(?)	(?)	Parallel	Parallel
Elongation	+	Indeter-minate		Indeter-minate	Indeter-minate	Indeter-minate	Indeter-minate	Indeter-minate	Indeter-minate	+	+
Figure	0	0	Fre-quent	Fre-quent	Usual	Usual	Rare	Fre-quent	Fre-quent	0	0
2 E	Indeter-minate	Indeter-minate		Indeter-minate	Indeter-minate	Large	Indeter-minate	Large	Indeter-minate	Indeter-minate	Indeter-minate
Sign	Indeter-minate	Indeter-minate	....	Indeter-minate	....	Indeter-minate	Indeter-minate	....	+	Indeter-minate	Indeter-minate

* From G. L. Keenan, *J. Biol. Chem.*, 1924, lxii, 163.

A systematic study of the optical properties of a number of amino acids has been made by Keenan.[131] The crystal habit, refractive indices, characters shown in parallel polarized light with crossed nicols, characters shown in converging polarized light with crossed nicols, and diagnostic characters of alanine, aspartic acid, cystine, glutamic acid, glycine, leucine, phenylalanine, serine, tryptophane, tyrosine, and valine were studied and Table 5 summarizes these data.

Ward[24] has measured the absorption of seven amino acids, *i.e.*, tryptophane, tyrosine, phenylalanine, alanine, histidine, glutamic acid, and cystine; only phenylalanine, tyrosine, and tryptophane gave marked absorption bands. From these as well as Kober's * results, it appears probable that the remaining amino acids show, as do glutamic acid and alanine, general absorption. According to Ward cystine is the only amino acid that has any marked absorption in the region of the solar ultraviolet light, which fact suggests that the presence of cystine in hair and wool, where the concentration approaches that of about 10 per cent, is of physiological importance in the protection of the organism against the harmful effects of prolonged exposure to sunlight.

SEPARATION BY ELECTROLYSIS

Advantage has been taken of the difference in reaction of the various amino acids to separate them electrolytically. Ikeda and Suzuki[132] have described a method for separating certain fractions of the products of protein hydrolysis by electrolysis. Foster and Schmidt[133] have more recently studied this method and have found it to be of practical importance.

On passing direct current through a solution of the protein cleavage products, which is placed in the center of a three-compartment cell, the amino acids are separated into three fractions consisting of (*a*) the amino acids which are predominantly acid, including aspartic and glutamic acids, which migrate to the anode, (*b*) the basic amino acids which include arginine, histidine, and lysine, which wander to the cathode, and (*c*) the remaining amino acids, which on account of the fact that their acid properties are about balanced by their basic properties, remain in the center compartment. The reactions in the different compartments are maintained at certain approximate pH values by the addition of $Ba(OH)_2$ or H_2SO_4. While this method will receive more detailed attention in the next chapter, it is of interest to note that when the reaction of the protein hydrolysate is more alkaline than pH 7.5,

[131] Keenan, G. L., *J. Biol. Chem.*, 1924, lxii, 163.
* Kober, P. A., *J. Biol. Chem.*, 1915, xxii, 433.
[132] Ikeda, K., and Suzuki, S., U. S. Patent No. 1015891, Jan. 30, 1912.
[133] Foster, G. L., and Schmidt, C. L. A., *Proc. Soc. Exp. Biol. Med.*, 1921-22, xix, 348.

histidine is not carried over into the cathode compartment, while at pH 5.5 analysis of the cathode solution shows the presence of the three basic amino acids in approximately the same proportions as in the original hydrolysate. This is not unexpected when it is recalled that the isoelectric point of histidine is considerably lower than that of either arginine or lysine.

DERIVATIVES

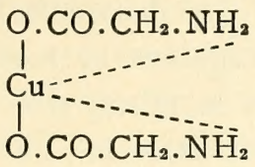
The amino acids, possessing both amino and carboxyl groups, are capable of forming a large variety of derivatives. Some derivatives are characteristic of all the amino acids, while others are specific for only certain ones. Where all the amino acids form the same derivative, the physical and chemical properties of the derivatives derived from the different amino acids may be very similar and again may be widely different. The derivatives are important in the detection of the presence or absence of certain amino acids, in removing certain of the amino acids from solution, in the isolation, preparation and purification of the individual acids, and in their quantitative determination.

By means of their carboxyl groups the amino acids are capable of all chemical reactions characteristic of this group. Thus, they unite with alkali metals, with bases and other basic compounds to form salts, they may be esterified, etc. Through their amino groups the amino acids form salts with acids and other acidic compounds, they may be benzoylated, acetylated, etc. A number of derivatives were mentioned under the section on color reactions. In the section on precipitants a large number of derivatives characterized by their insolubility in ordinary solvents were discussed.

For proving the presence of certain of the amino acids, the following derivatives, which usually form definite crystalline compounds, are made: copper salt, picrate, ethyl ester hydrochloride, α-naphtholisocyanate, benzoylate, β-naphthalene sulfonic chloride, occasionally the 4-nitrotoluol-2-sulfonic chloride, picrolonate, double salts of the heavy metals such as phosphotungstate, chloroplatinate, gold chloride and nitrate, silver nitrate, mercuric chloride, etc. Salts of other metals such as barium, calcium, strontium, nickel, zinc, and cadmium; also salts of phosphomolybdic, tannic, hydrochloric, nitric, sulfuric, and oxalic acids are often used in the identification of the amino aids. Esters of alcohols and their hydrochlorides are also used.

Copper Salt. Most of the ordinary derivatives used for the purpose of identification are easily prepared and purified. The copper salts of most of the amino acids are readily formed when an aqueous solution

of the amino acid is boiled wih copper oxide, hydroxide, carbonate, or acetate. The salts may ordinarily be crystallized out of a hot aqueous solution. The formula for copper glycine is $(NH_2.CH_2.COO)_2Cu + H_2O$, which perhaps has the constitution represented by



Ninhydrin. Numerous derivatives serve for the characterization of the individual amino acids. Glycine, as well as all other known α-amino acids (amino acids containing an imino instead of an amino group, such as proline and hydroxyproline, excepted), give when boiled in an aqueous solution with triketohydrindene hydrate (ninhydrin)* a blue color.[66, 67]

Picrates. The picrate is prepared by mixing a concentrated aqueous solution of the amino acid with about a fourfold quantity of picric acid dissolved in alcohol. The picrate crystallizes on cooling. The formula for glycine picrate is $C_6H_3O_7N_3(NH_2.CH_2.COOH)$.

Ethyl Esters. The ethyl ester hydrochlorides of the amino acids may be prepared by esterifying the free amino acids or their hydrochlorides with alcohol and gaseous hydrochloric acid. The formula for glycine ethyl ester hydrochloride is $HCl.NH_2.CH_2.COOC_2H_5$, which crystallizes in long colorless needles of m.p. 144°. It is very soluble in water, difficultly soluble in alcohol and very difficultly soluble in ether.

α-Naphthol-isocyanates. The α-naphthol-isocyanate is formed when the amino acid is coupled with α-naphthol-isocyanate in alkaline solution by means of acid. The formula for the glycine derivative is $C_{10}H_7NH.CO.NH.CH_2.COOH$. It is soluble in warm alcohol, very difficultly soluble in water, soluble in alkalies and ammonia.

Benzoylates. The benzoyl derivatives of the amino acids are prepared in a manner similar to the preparation of other benzoyl derivatives. The benzoyl derivative of glycine (hippuric acid) may be prepared in good yield by the following method: An aqueous solution of glycine is made slightly alkaline with sodium hydroxide; equimolecular quantities of benzoyl chloride (a slight excess of this reagent is beneficial) and sodium hydroxide are then added alternately in small portions. The mixture is shaken at room temperature until the odor of benzoyl chloride disappears. The solution is acidified with hydrochloric acid and after cooling the hippuric and benzoic acids may be filtered off. After

* The nature of these derivatives is discussed by Retinger, J. M., *J. Am. Chem. Soc.*, 1917, xxxix, 1059.

drying the benzoic acid may be dissolved in warm petroleum ether. The hippuric acid is then recrystallized from water. The formula for the benzoyl derivative of glycine is $C_6H_5.CO.NH.CH_2.COOH$ (hippuric acid).

β-Naphthol-sulfonates. The β-naphthol-sulfonic chloride derivative is prepared [110] by shaking together at room temperature two mols of β-naphthol-sulfonic chloride in ether, one mol of the amino acid and one mol of normal sodium hydroxide. Three times at hourly intervals an equal quantity of normal sodium hydroxide is added. The ether layer is separated from the aqueous one, clarified with animal charcoal, and acidified with hydrochloric acid. A crystalline precipitate immediately appears. The glycine derivative has the formula $C_{10}H_7.SO_2.NH.CH_2.COOH$.

4-Nitrotoluol-2-sulfonates. The 4-nitrotoluol-2-sulfonic derivative [134] is prepared in a manner similar to any sulfonic chloride derivative, such as the benzene-sulfonic chloride derivatives. Oseki [135] has made a special study of these derivatives.

Carbamino Salts. Many of the derivatives of the amino acids are used as a means of quantitatively determining some of the amino acids. The ethyl ester hydrochlorides, for example, all have different boiling points and therefore may be separated by fractional distillation. This is the basis of Fischer's esterification method for the isolation and determination of the various amino acids. Another series of derivatives which is important, not only from this point of view, but because they possibly exist in the body, are the carbamino derivatives of Siegfried.[136] The salts of the carbamino acids usually formed are the calcium salts, which are insoluble in alcohol, forming crystalline granules. These are soluble in water forming a clear solution and on boiling calcium carbonate is separated and the amino acid is freed. The calcium salts have the following structure:

$$\begin{array}{l} R.CH\text{——}CO.O \\ \quad\;\; | \qquad\quad\; | \\ \quad NH.CO.O.Ca \end{array}$$

Derivatives with Ammonia. For a study of the derivatives resulting from the combination of the amino acids with ammonia we are indebted to Bergell and his co-workers.[137] He obtained the acid amides or the amide hydrochloride or hydrobromide of a number of the amino acids.

[134] Siegfried, M., *Z. physiol. Chem.,* 1905, xliv, 85.
[135] Oseki, T., *J. Tokyo Chem. Soc.,* 1920, xli, 8, cited through *Chem. Abstracts,* 1920, xiv, 2780.
[136] Siegfried, M., *Z. physiol. Chem.,* 1905, xliv, 85; 1906, xlvi, 401; *Ergebn. Physiol.,* 1910, ix, 334; Siegfried, M., and Neumann, C., *ibid.,* 1908, liv, 423; Siegfried, M., and Liebermann, H., *ibid.,* 1908, liv, 437; Siegfried, M., and Schutt, E., *ibid.,* 1912, lxxxi, 260.
[137] Bergell, P., and Feigl, J., *Z. physiol. Chem.,* 1908, liv, 258; Bergell P., and von Wülfing, H., *ibid.,* 1910, lxiv, 348; Bergell, P., and Boll, P., *ibid.,* 1912, lxxvi, 464.

Glycinamide hydrochloride, $NH_2.CH_2.CONH_2.HCl$, for example, was prepared by the action of ammonia on chloracetamide in the cold.

Derivatives with Lipoids and Sugars. On the assumption that it is quite possible that there are compounds in the tissues of plants and animals, in the structure of which amino acids take part together with representatives of the lipoid and carbohydrate series, and that knowledge of definite compounds of this type would be of considerable importance, Abderhalden and his co-workers have prepared and studied an elaborate series of types of compounds containing amino acids and representatives of lipoids and carbohydrates. Abderhalden and Guggenheim [138] studied the condensation products from glycerol and amino acids and obtained compounds such as glycerol-monotyrosine ether.

Abderhalden and Funk [139] studied the production of compounds of the amino acids and fatty acids by the same method which Emil Fischer used for the formation of polypeptides, except that they used in the place of the halogenacyl chlorides the unsubstituted fatty-acid chlorides. The carboxyl of the fatty acid combines with the amino group of the amino acid as follows:

$$R.COCl + HNH.CH_2.COOH = RCH_2.CO.NH.CH_2.COOH + HCl$$

The compounds prepared were palmityl-glycine, palmityl-*d*-alanine, mono-palmityl-*l*-tyrosine, palmityl-ether of palmityl-*l*-tyrosine, palmityl-ether of palmityl-3-5-diiodo-*l*-tyrosine, stearyl glycine, stearyl-*d*-alanine, and stearyl-ether of stearyl-*l*-tyrosine.

Continuing along the same lines, Abderhalden and Kautzsch [140] prepared some derivatives of cholesterol with fatty-acid chlorides. The compounds prepared were *α*-bromisovaleryl-cholesterol, chloracetyl-cholesterol, *α*-bromisocapronyl-cholesterol, glycyl-cholesterol-chlorhydrate, glycyl-cholesterol, isobutyryl-cholesterol, isovaleryl-cholesterol, lauryl-cholesterol, palmityl-cholesterol, stearyl-cholesterol, dichloracetyl-resorcin, dichloracetyl-pyrocatechin, and dichloracetyl-hydrochinone.

Still more complicated derivatives of glycerol and the amino acids were prepared by Abderhalden and Baumann,[141] such as glycerol-di-(glycyl-*l*-tyrosine) ether, glycerol-dityrosine ether, and glycerol-trityrosine ether.

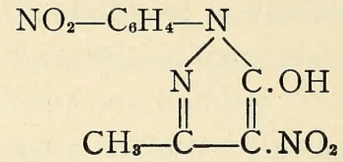
Analogous compounds with sugars have been prepared by Kostytschew and Brilliant.[142]

Urea Derivatives. The derivatives resulting from the reaction of

[138] Abderhalden, E., and Guggenheim, M., *Z. physiol. Chem.*, 1910, lxv, 53.
[139] Abderhalden, E., and Funk, C., *Z. physiol. Chem.*, 1910, lxv, 61.
[140] Abderhalden, E., and Kautzsch, K., *Z. physiol. Chem.*, 1910, lxv, 69.
[141] Abderhalden, E., and Baumann, L., *Z. physiol. Chem.*, 1911, lxxii, 50.
[142] Kostytschew, S., and Brilliant, W., *Z. physiol. Chem.*, 1914, xci, 372; 1923, cxxvii, 224; *Bull. de l'Acad. Sci. de St. Petersbourg*, 1916, 953.

urea with the amino acids have been studied by Lippich.[143] Carried out in the presence of barium hydroxide, the reaction seems to be of importance, since the resulting derivatives are readily isolated and identified. Lippich [144] has applied the uramino acid reaction to the isolation of leucine from blood serum, but the technic was not altogether satisfactory.

Picrolonates. The picrolonates resulting from the union of picrolonic acid and the amino acids are important not only as a means of identification, but also as a means of separating certain amino acids. Picrolonic acid,



was first introduced by Knorr [117] as a precipitant for organic bases. Steudel [118] applied it to the hexone bases obtained by the hydrolysis of proteins. Mayeda [119] has described the picrolonates of the aromatic amino acids, tryptophane, and phenylalanine. Abderhalden and Weil [120] studied the picrolonates of alanine, glycine and inactive leucine. Levene and Van Slyke [121] have described the picrolonates of the monoamino acids. Most of the picrolonates are beautiful crystalline definite salts, fairly insoluble in cold water. Many of them decompose when they melt. In Table 2 (p. 34) the physical properties of the picrolonates of several of the amino acids are included. One advantage of the picrolonates as a means of isolation of the amino acids is that the free amino acids can be readily and quantitatively regained from them.[121]

Phosphotungstates. One of the most widely used derivatives of the amino acids are the phosphotungstates. While these derivatives have been used for identification purposes, they have been found more valuable for purposes of isolation of certain of the amino acids, especially the basic ones. Phosphotungstic acid was introduced as a precipitant for organic bases by Drechsel.[145] Its preparation and purification have also been described by Winterstein.[146] Gulewitsch [147] has described the preparation and formula of arginine phosphotungstate and Barber [148] has described the phosphotungstates of glycine, alanine, glutamic acid, and aspartic acids. Wechsler [149] has described the arginine and histidine

[143] Lippich, F., *Z. physiol. Chem.*, 1914, xc, 124.
[144] Lippich, F., *Z. physiol. Chem.*, 1914, xc, 145.
[145] Drechsel, E., *Ber. chem. Ges.*, 1887, xx, 1452.
[146] Winterstein, E., *Chem. Ztg.*, 1898, xxii, 539.
[147] Gulewitsch, W., *Z. physiol. Chem.*, 1899, xxvii, 178.
[148] Barber, M., *Monatsh.*, 1906, xxvii, 379.
[149] Wechsler, E., *Z. physiol. Chem.*, 1911, lxxiii, 138.

derivatives, while Levene and Beatty [150] have studied the phosphotungstate derivatives of several of the simpler amino acids. Finally Drummond [151] has studied especially the solubilities of a number of the amino acid phosphotungstates.

Salts of Metallic Elements. The amino acids readily form salts with the metallic elements. As has been mentioned, of all the derivatives for identification purposes, the copper salt is one of the best. The following metallic derivatives have been described: [152] the silver, mercury, lead, palladium, copper, nickel, cobalt, zinc, cadmium, strontium, barium and magnesium salts of glycine; the copper and nickel salts of alanine; the copper salt of valine; the copper, zinc, silver, mercury, and lead salt of leucine; the copper, cobalt, silver, and nickel salt of isoleucine; the sodium, calcium, barium, mercury, lead, and silver salt of aspartic acid; the sodium, potassium, calcium, barium, silver, copper and lead salts of glutamic acid; the silver salt of arginine; the copper, mercury, silver, lead and cadmium salts of cystine; the copper salt of phenylalanine; the copper, silver, barium, lead, sodium, calcium, and mercury salts of tyrosine; the copper, sodium, and silver salts of tryptophane; the sodium and silver salts of histidine; and the copper salt of proline. Harris [153] especially has studied the metallic derivatives of cysteine. He prepared the iron, manganese, copper, cobalt, nickel, chromium, bismuth, tin, and mercury salts of this substance.

Neutral Salts. Pfeiffer and his co-workers [113] have made an extensive study of the products resulting from the addition of neutral salts to the amino acids. They have studied particularly the addition products of the halides of the alkali and alkaline earth metals with glycine and alanine. They have obtained definite compounds of glycine with barium chloride, strontium chloride and bromide, calcium chloride and bromide, and with magnesium and lithium chlorides. (See section on Precipitants.)

For some purposes connected with their work regarding the metabolism of cystine, Shiple and Sherwin [154] prepared the following compounds: Cystine, diphenylacetyl cystine, phenyluramino cystine, cystine phenylhydantoin, cysteine-hydrochloride, benzyl cysteine, phenylacetyl benzyl cysteine, phenyluramino benzyl cysteine, benzyl cysteine phenylhydantoin, acetyl benzyl cysteine, *p*-chloro-benzyl cysteine, phenyluramino cystine, and *p*-bromophenyl mercapturic acid. Some physical and chemical properties of these compounds are given.

[150] Levene, P. A., and Beatty, W., *Z. physiol. Chem.*, 1906, xlvii, 149.
[151] Drummond, J. C., *Biochem. J.*, 1918, xii, 5.
[152] Abderhalden, E., "Biochemisches Handlexikon," Vol. IV (Berlin), 1911.
[153] Harris, L. J., *Biochem. J.*, 1922, xvi, 739.
[154] Shiple, G. J., and Sherwin, C. P., *J. Biol. Chem.*, 1923, lv, 671.

Halogen Derivatives. Known derivatives of the amino acids and the halogens are few in number. Di-iodotyrosine has been referred to. Although bromine adds to histidine, tyrosine, and to tryptophane, few derivatives have been isolated. Cystine reacts with bromine, but it is probable that this is a case of oxidation instead of addition. Plimmer and Phillips [155] have shown that histidine absorbs two atoms of bromine, but no derivatives have been isolated.

Hydantoins. All the optically active α-amino acids of the general type,

$$\begin{array}{l} \mathrm{R.CH.NH_2} \\ \quad | \\ \mathrm{COOH,} \end{array}$$

are readily converted by the action of potassium cyanate into the salts of the corresponding uramino acids,

$$\begin{array}{l} \mathrm{R.CH.NH.CO.NH_2,} \\ \quad | \\ \mathrm{COOH} \end{array}$$

and then by the action of boiling dilute mineral acids are transformed into the corresponding hydantoins,

$$\begin{array}{l} \mathrm{R.CH.NH.CO,} \\ \quad | \qquad\quad | \\ \mathrm{CO\text{——}NH} \end{array}$$

by the loss of a molecule of water. The hydantoins usually form well-crystallized compounds with definite melting points. They are sparingly soluble in water, but form soluble salts. They are often used for purposes of identification.

The hydantoins are also of interest because a conjugation of carbamic acid, NH_2COOH, with amino acids to form uramino acids or their anhydrides, the hydantoins, have been observed in several cases, as after feeding sarcosin, aminobenzoic acid, phenylalanine, taurine, and tyrosine. Also the uramino acids can be easily produced as transformation products from urea in the concentration of urine by the aid of heat.[156] It will be noted that the ring in hydantoin itself,

$$\begin{array}{l} \mathrm{CH_2.NH.CO_3} \\ \quad | \qquad\quad | \\ \mathrm{CO\text{——}NH} \end{array}$$

except for the double bonds, is the imidazole ring and thus shows a possible relationship to histidine.

Methylation. For purposes of identification, separation, and determination, Engeland and his co-workers have made use of methylated

[155] Plimmer, R. H. A., and Phillips, H., *Biochem. J.*, 1924, xviii, 312.
[156] Lippich, F., *Ber. chem. Ges.*, 1908, xli, 2953, 2974; Dakin, H. D., *J. Biol. Chem.*, 1910, viii, 25.

derivatives (see page 83). The method of the isolation of the monoamino acids, as originally described by Engeland,[157] consists in treating them with potassium and methyl iodide in excess at as low a temperature as possible, so as to avoid the production of complex compounds of high molecular weights. These latter are always formed if the methylation is conducted under pressure, even at 80 degrees, and their quantity is greater the higher the temperature. The reaction is carried out in methyl alcohol solution. The methylated products are then separated by means of the varying solubilities of their derivatives with metallic salts, such as gold or mercuric chloride. Before methylation the mixture is treated with phosphotungstic acid, to remove the diamino acids, and from the filtrate glutamic acid, leucine, and tyrosine are separated by crystallization, the first in the form of the hydrochloride. The method as such is not applicable to the separation of the diamino acids. The results of the separation are regarded by him as essentially quantitative.

The methylation of phenylalanine and glutamic acid was described by Engeland [158] and of histidine, arginine, and lysine by Engeland and Kutscher; [159] Abderhalden and Schwab [160] have described the methylation of tyrosine.

REACTIONS *

Possessing both carboxyl and amino groups, the amino acids behave both as acids and as bases. The reaction of all monoamino-monocarboxylic acids is amphoteric to litmus; the diamino acids and histidine and arginine react alkaline in solution; while the monoamino-dicarboxylic acids, *i.e.*, aspartic and glutamic acids, are acid in reaction.

They form two series of salts. As bases they react like substituted ammonias to form hydrochlorides with hydrochloric acid. The salts thus formed ionize into the amino acid as the cation and chlorine as the anion. As acids they unite with bases, such as sodium hydroxide, to form the sodium salt of the amino acid, which on ionizing yields sodium as the cation and the amino acid as the anion. As they are both weak acids and weak bases, both of their salts undergo hydrolytic dissociation, so that the sodium salts are alkaline in reaction, due to the formation of

[157] Engeland, R., *Ber. chem. Ges.*, 1909, xlii, 2962.
[158] Engeland, R., *Ber. chem. Ges.*, 1910, xliii, 2662.
[159] Engeland, R., and Kutscher, Fr., *Z. Biol.*, 1912, lix (N.S. xli), 415
[160] Abderhalden, E., and Schwab, E., *Z. physiol. Chem.*, 1925, cxlviii, 17.

* In a recent paper [*J. Biol. Chem.*, 1928, lxxvii, 91 (1926)] Dakin and West describe in interesting reaction which appears to be general for the α-amino acids. When the amino acids are warmed with acetic anhydride and pyridine, carbon dioxide is given off and two acetyl groups are introduced, one on a nitrogen atom and one on a carbon atom. The compounds formed are derivatives of acetylaminoacetone having the general formula $R.CH.(NH.COCH_3).CO.CH_3$. These ketones form crystalline phenylhydrazones of definite melting points and these may be used for purposes of identification. Reference is also made to the possible uses of the alkyl acetaminoacetones for the preparation of pharmacologically active substances. The possible biological significance of the reaction is also discussed.

sodium hydroxide; and the hydrochlorides are acid, due to the formation of a small amount of hydrochloric acid.

The amino acids are capable of entering into a large variety of chemical reactions. By means of their carboxyl groups, the amino acids are capable of the chemical reactions characteristic of this group. Thus they unite with metals, with bases, and other basic compounds to form salts; they may be esterified, etc. By means of their amino groups, the amino acids form salts with acids and other acidic compounds; they may be methylated, acetylated and benzoylated; they form compounds with α-naphthol-isocyanate, β-naphthalene sulfonic chloride, and nitrotoluene sulfonic chloride; etc. The amino acids form double salts with phosphotungstic, phosphomolybdic, and chloroplatinic acids; with salts of the heavy metals, such as gold, silver, mercury and lead; and with neutral salts of many other metals, such as copper, cadmium, zinc, barium, calcium, lithium, etc.

Many reactions characteristic of certain groupings, some of which are natural to all the α-amino acids while some are characteristic of certain amino acids, have been described in the section on color reactions. A large number of reactions resulting in the formation of insoluble compounds with the amino acids were also described in the section on precipitants for the amino acids. In the section on derivatives a number of reactions in which compounds of definite composition and other properties suitable for identification purposes were described.

Some of the reactions referred to in these sections have also been used for purposes of quantitative estimation of certain amino acids. Folin and Denis' sodium tungstate-phosphomolybdic acid reagent for the detection of tyrosine has been developed into a quantitative method for the estimation of this amino acid. The ability of certain amino acids to be brominated lends itself to the quantitative estimation of certain amino acids also. Voisenet's formaldehyde-nitrite color reaction for the detection of tryptophane has been used for the quantitative determination of this amino acid. The ethyl ester hydrochlorides of Fischer have been mentioned as being excellent derivatives for the isolation and detection of the amino acids, and Fischer's method, while cumbersome, is still, perhaps, one of the most reliable methods for the determination of the amino acid content of any protein. The use of phosphotungstic acid has also been mentioned as a reaction which has been made use of in connection with the identification and separation of the hexone bases. In addition to these reactions there are others which are of interest either from a purely chemical or from a biological point of view.

1. *Condensation of Aldehydes with the Amino Acids.* The amino acids, through their amino groups, condense with formaldehyde, giving as a final product a methylene substitution compound:

$$\begin{array}{l} R.CH.COOH \\ | \\ NH_2 \end{array} + H.CHO \longrightarrow \begin{array}{l} R.CH.COOH \\ | \\ N = CH_2 \end{array} + H_2O$$

By this reaction the methylene substitution products are formed. The substitution of the methylene group for the positive element hydrogen in the amino group reduces the basicity of the amino group so that the acid character of the carboxyl comes clearly into evidence, and these substituted acids may be titrated with sodium hydroxide, using phenolphthalein as indicator. This reaction is extremely important, partly because these substitution products by reduction go over readily into the methyl amino derivatives,

$$\begin{array}{l} R.CH.COOH \\ | \\ N = CH_2 \end{array} + H_2 \longrightarrow \begin{array}{l} R.CH.COOH \\ | \\ H—N—CH_3 \end{array}$$

which are found in animals and plants and are possibly produced in this way, and partly because this reaction is the basis of Sörensen's method for the determination of the amount of total amino acids in a mixture.

2. *The Carbamino Reaction of the Amino Acids.* An interesting and important reaction of the amino acids is their union with calcium and carbonic acid to form carbamino compounds, a reaction discovered and studied by Siegfried.[136] Just as ammonia and carbonic acid unite to form ammonium carbonate and ammonium carbamate, the ammonium salt of carbamic acid, $NH_4OCO.NH_2$, the amino acids, through their amino groups, unite with carbonic acid in a similar manner to form carbamino acids. The salts of the carbamino acids usually formed are the calcium salts, which are insoluble in alcohol, forming crystalline granules. These are soluble in water to a clear solution and, on boiling, calcium carbonate is separated and the amino acid is freed. The calcium salts have the following structure:

$$\begin{array}{l} R.CH—CO.O \\ | \quad\quad | \\ NH.CO.OCa \end{array}$$

This reaction has been used in getting an idea of the composition of mixtures of amino acids and in studying the course of the hydrolytic decomposition of the proteins. This is done by first determining the nitrogen in the aqueous solution containing the calcium salt, then boiling to precipitate the calcium carbonate, filtering and determining the

amount of calcium carbonate and from that the combined carbonic acid. There is thus obtained a relation between the number of nitrogen atoms and the molecules of carbon dioxide which have been in union. The result is expressed as the quotient CO_2/N. In the case of monoamino acids this quotient will be one. Such a quotient will mean that all the amino acids in the mixture are monoamino acids and all the amino groups are free, since the reaction only occurs between free amino groups and free carboxyl groups. If diamino acids are present, or if the amino acids are in part combined in peptide unions, the quotient will be less than one, since the amount of nitrogen is greater than the number of free amino groups. It is possible by studying this quotient to follow the course of the digestion of the proteins and determine when the reaction is complete.

3. *Reaction with Nitrous Acid.* The reaction between aliphatic amino groups and nitrous acid with the production of an alcohol and gaseous nitrogen has long been known. Since the nitrogen is in gaseous form it leaves the system and the reaction goes to completion in that direction, or to the right in the following equation:

$$\begin{array}{l} \mathrm{R.CH.COOH} \\ \quad | \\ \quad \mathrm{NH_2} \end{array} + \mathrm{HO.NO} \longrightarrow \begin{array}{l} \mathrm{R.CH.COOH} \\ \quad | \\ \quad \mathrm{OH} \end{array} + \mathrm{N_2} + \mathrm{H_2O}$$

Van Slyke [161] has made use of this reaction in his well-known method for the determination of the aliphatic amino groups in protein hydrolysates.

4. *Deaminization.* The amino group of the amino acids is detached only with great difficulty in acid hydrolysis, or by the actions of alkalies. The amino acids are most stable in the form of their acid salts. Most of them are quite stable, also, in alkaline solutions, although cystine and cysteine will lose their sulfur to a large extent in alkaline solution and arginine decomposes into ornithine and urea. The fact that the amino nitrogen is so firmly attached and the amino acids so stable in acid solution enables the conclusion to be drawn that the ammonia, which always appears in small amounts when a protein is hydrolyzed in acid solution, cannot have come from the amino acids, but must have had some other linkage in the protein molecule. It evidently was combined in a free carboxyl group and represents acid amide nitrogen and not amino acid nitrogen. But while the amino group is not readily detached by simple hydrolysis, it can be readily removed by oxidation.

(*a*) *Oxidation to Ketonic Acids.* By various oxidizing agents, such as hydrogen peroxide and permanganate, under the proper conditions,

[161] Van Slyke, D. D., *J. Biol. Chem.*, 1911, ix, 185; 1911, x, 15; 1915, xxii, 281.

the amino group is displaced and the corresponding ketonic acid is formed. This reaction is reversible. The reaction may be written as follows:

$$\begin{matrix} R.CH.COOH \\ | \\ NH_2 \end{matrix} + O \rightleftharpoons R.CO.COOH + NH_3$$

The reaction in the left direction goes on only in the presence of reducing agents. Both these reactions are of great importance, since they occur in the metabolism of amino acids in living matter and may be involved in the synthesis of amino acids from the decomposition products of the sugars and ammonia; they also show how the proteins may be converted, with the loss of ammonia, into carbohydrates and fats.

(*b*) *Oxidation to Aldehydes.* The oxidation of α-amino acids to aldehydes may be effected with a variety of reagents including hydrogen peroxide,[162] lead oxide and dilute sulfuric acid,[163] alloxan,[164] glyoxals,[165] and sodium hypochlorite.[166] In all these and similar reactions, the nitrogen from the α-amino acid seems to be very easily removed by oxidation. Langheld expressed the oxidation as follows:

$$\begin{matrix} R.CH.COOH \\ | \\ NH_2 \end{matrix} \xrightarrow{+\ NaClO} \begin{matrix} R.CH.COONa \\ | \\ NHCl \end{matrix} \xrightarrow{+\ H_2O} R.CHO + NH_3 + NaCl + CO_2$$

Although the amino group is replaced by oxygen with ease, it is not readily replaceable with hydrogen. That is, if a protein is hydrolyzed with hydrochloric acid and tin, so that nascent hydrogen is set free, the hydrogen does not displace the amino group to form the fatty acid. In fact, tin is not infrequently added to reduce the decomposition caused by oxidation during a hydrolysis.

5. *Formation of Lactams and Piperazine Nuclei.* Another important peculiarity of the α-amino acids is the fact that they form anhydrides, two molecules combining, with the greatest readiness. Thus, it is only necessary to evaporate solutions of leucine or other amino acids to produce some condensation to imides, such as leucinimide; diketopiperazine nuclei are thus formed:

$$\begin{matrix} (CH_3)_2{=}CH{-}CH_2{-}CH.NH{-}CO \\ \qquad\qquad\qquad\qquad\quad | \qquad\quad | \\ \qquad\qquad\qquad\qquad OC{-}NH{-}CH{-}CH_2{-}CH{=}(CH_3)_2. \end{matrix}$$

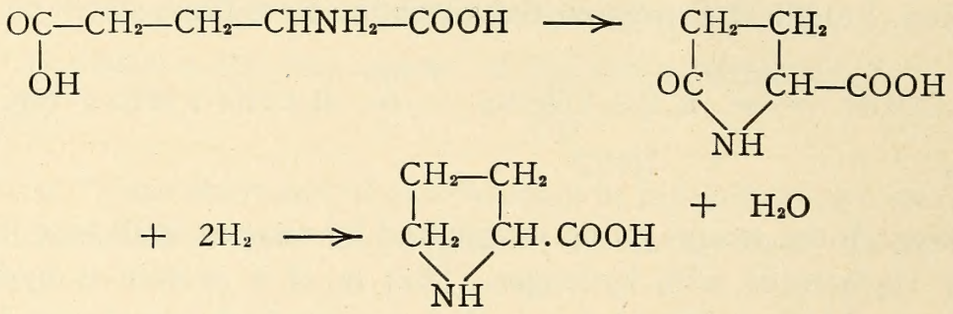
Leucinimide (*Diisobutyl-diketopiperazine*)

[162] Dakin, H. D., *J. Biol. Chem.*, 1906, i, 171.
[163] Liebig, J., *Ann. Chem.*, 1849, lxx, 311.
[164] Strecker, A., *Ann. Chem.*, 1862, cxxiii, 363, cited through Hurtley, W. H., and Wootton, W. O., *J. Chem. Soc., Trans.*, 1911, xcix, 288.
[165] Dakin, H. D., *J. Biol. Chem.*, 1914, xviii, 29.
[166] Langheld, K., *Ber. chem. Ges.*, 1909, xlii, 392, 2360.

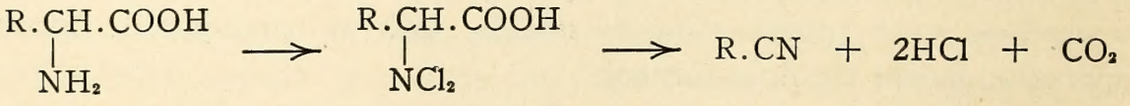
It is considered probable that heterocyclic rings of this kind occur in the protein molecule, but their presence has not yet been proved. Such a condensation may, however, give rise to some of the cyclic amino acids from straight chain amino acids.

When the amino group is in the α or δ position as compared with the carboxyl, as it is in glutamic acid, for example, $COOH.CH_2.CH_2.CHNH_2.COOH$, an internal condensation within the molecule occurs with the greatest ease. These anhydride substances correspond to lactones and are called lactams. It is an interesting fact that although the amino acids themselves are without special physiological or toxic action, these lactams are powerful poisons, producing strychnine-like convulsions. By this lactam formation some of the cyclic amino acids may be formed from the straight chain amino acids.

This may, for example, be the origin of proline. Glutamic acid undergoes a condensation of this kind to form the lactam, pyrrolidone carboxylic acid, which by reduction will yield pyrrolidine carboxylic acid, or proline.



6. *Oxidation to Cyanides.* Dakin [167] has shown that by less vigorous oxidation than is used to form aldehydes, the amino acids may be oxidized to cyanides. He made use of the same reagent that Dakin, Cohen, Daufresne, and Kenyon [168] used to oxidize the amino acids to aldehydes, *i.e.*, sodium-paratoluene-sulfochloramide. The formation of cyanides from the amino acids by oxidation is illustrated by Dakin as follows:



Dakin obtained traces of hydrocyanic acid from glycine, acetonitrile from alanine, isobutyl cyanide (isovaleronitrile) from leucine and cyanobenzene (benzonitrile) from amino phenyl acetic acid. The yields in the case of the last three amino acids were large.

7. *Interconversion of α-Amino Acids, α-Hydroxy Acids and*

[167] Dakin, H. D., *Biochem. J.*, 1916, x, 319.
[168] Dakin, H. D., Cohen, J. B., Daufresne, M., and Kenyon, J., *Proc. Roy. Soc.* (*London*), B., lxxxix, 1916, 232.

α-Ketonic Aldehydes.[169] Because of the possibility that substances belonging to the group of glyoxals may play a rôle in the intermediary metabolism of proteins and carbohydrates, the reactions showing the interconversion of the α-amino acids, α-hydroxy acids, and α-ketonic aldehydes are important. Dakin and Dudley have shown that α-amino and α-hydroxy acids yield glyoxals when digested at body temperature with substances such as *p*-nitrophenylhydrazine, which form insoluble derivatives with the glyoxals.

$$R.CHNH_2.COOH \longrightarrow R.CO.CHO + NH_3$$
$$R.CHOH.COOH \longrightarrow R.CO.CHO + H_2O$$

Glyoxals thus formed may be reconverted into α-hydroxy acids by the action of the widely distributed enzymes, glyoxalases. Methyl glyoxal, for example, yields lactic acid:

$$CH_3.CO.CHO + H_2O \longrightarrow CH_3.CHOH.COOH$$

Methyl glyoxal may be readily formed *in vitro* from glucose and from lactic acid and alanine. Conversely when methyl glyoxal or *l*- or *d*-lactic acid, which are formed from it by the action of glyoxalase, or *d*- or *l*-alanine, are given to a dog rendered glycosuric by phlorhizin, they are all converted into glucose. Methyl glyoxal, therefore, may be regarded as a possible intermediary stage in the interconversion of glucose, lactic acid and alanine. Finally it has been shown that a glyoxal, such as phenyl glyoxal, may yield the corresponding α-ketonic acid on perfusion through a surviving liver. By combining the results obtained from many different types of experiments, it appears that α-amino and α-hydroxy acids might be regarded as being in equilibrium with the corresponding glyoxals and that the latter substances might be intermediate stages in the interconversion of amino and hydroxy acids. The relationship may be crudely represented as follows:

$$R.CHOH.COOH \rightleftharpoons R.CO.CHO \rightleftharpoons R.CHNH_2.COOH$$

8. *Exhaustive Methylation of the Amino Acids.*[157, 158, 159] In the Sörensen method for the determination of total amino acids the basicity of the amino group is reduced by the formation of the methylene substitution derivatives by treating the amino acids with formaldehyde so that the carboxyl groups may be titrated with standard alkali. For the purpose of separation and determination, Engeland recommends strengthening the basicity, since organic bases are in general easier to separate and to obtain analytically than the organic acids. For this purpose Engeland submits the amino acids to exhaustive methylation.

[169] Dakin, H. D., and Dudley, H. W., *J. Biol. Chem.*, 1913, xiv, 555; 1913, xv, 127; 1914, xviii, 29.

Griess,[170] who first applied this reaction to amino acids, obtained betaine from glycine. The method may be illustrated by the following reaction:

$$\begin{array}{l} H.CH.NH_2 \\ \quad | \\ \quad COOH \end{array} + 3CH_3I + 3NaOH = \begin{array}{l} H.CH.N(CH_3)_2 \\ \quad | \quad\quad | \\ \quad CO{-}O \quad CH_3 \end{array} + 3NaI + 3N_2O$$

The method of exhaustive methylation can be applied with advantage to a mixture of amino acids. The corresponding betaines can be separated by the different solubilities of their double salts with mercury, platinum, and gold chlorides. These double salts are crystalline and may be used for purposes of identification. Vickery states, however, that betaines are not easily separated.*

9. *Action of Hypochlorites on Amino Acids.* While sodium hypochlorite has been referred to as an oxidizing agent for the purpose of oxidizing amino acids to aldehydes,[166] Wright [171] has found that the hypochlorites also may act as chlorinating agents. Glycine was found to form a stable dichloroaminoacetic acid while cystine, as well as most other amino acids examined, forms very unstable derivatives.

SYNTHESIS OF POLYPEPTIDES

Although Liebig was the first to regard proteins as combinations of amino acids, no definite theory of the mode of combination of the amino acids in a protein molecule seems to have been proposed before the beginning of the twentieth century. In 1902 Hofmeister [172] presented an excellent summary and criticism of the knowledge of the protein molecule to date. The following summary, with a few additions by the authors, is a condensed form of the material given in Plimmer's Monograph on "The Chemical Constitution of the Proteins." [173]

There are three ways in which we can conceive that the amino acids are combined together in the protein molecule: (*a*) The carbon atoms can be linked together directly; (*b*) the carbon atoms can be linked together by an oxygen atom, and (*c*) the carbon atoms can be linked together by a nitrogen atom.

Of several different possible arrangements of combining two carbon atoms by a nitrogen atom, the combination $-CH_2-NH-CO$ is most likely because of the following significant facts:

(*a*) A small proportion of the total nitrogen of the protein molecule is liberated on hydrolysis as ammonia; this points to the presence of this

[170] Griess, P., *Ber. chem. Ges.*, 1875, viii, 1406.
* Vickery, H. B., private communication.
[171] Wright, N. C., *Biochem. J.*, 1926, xx, 524.
[172] Hofmeister, F., *Ergebn. Physiol.*, 1902, i, Part I, 759.
[173] Plimmer, R. H. A., "The Chemical Constitution of the Proteins," Part II, Longmans, Green & Co., 1913, p. 27-43.

linking in the form of acid amide, $-CO-NH_2$, combinations. A very large percentage of the total nitrogen in a protein hydrolysate is in the form of amino (NH_2) nitrogen. The amino groups are not all present as such in the protein molecule because on treating a protein with nitrous acid the amount of nitrogen liberated is very small compared to that obtained by similar treatment of the protein hydrolysate. These facts are best explained by assuming that most of the amino groups ($.NH_2$) of the end-products of protein hydrolysis exist in the protein molecule as imino ($:NH$) groups.

(*b*) The biuret reaction, which is one of the chief characteristics of a protein, is, according to Schiff, given by those substances which contain two CO—NH complexes, or two CS—NH or C(NH)—NH complexes, and under certain conditions two $-CH_2-NH$ complexes, combined together directly, or by a carbon atom, or by a nitrogen atom, as, for example, in oxamide, malonamide, biuret, glycine amide, sarcosine amide, and aspartic acid diamide. All these compounds give very intense biuret reactions and therefore the presence of —NH—CO—CH—NH— groups in the protein molecule is very probable.

(*c*) The combination of amino acids by the formation of CH_2—NH—CO groups is supported by the facts observed in the living body. Hippuric acid, $C_6H_5.CO.NH.CH_2.COOH$, is formed from benzoic acid and glycine by the kidney, and the bile acids are presumably substances of similar constitution.

(*d*) The condensation products of amino acids, obtained by Schaal, Grimaux, and by Curtius, many of which give the biuret reaction, support the above contention.

Before the researches of Emil Fischer and his pupils established the predominant linkage in the protein molecule, much important knowledge was obtained through the studies of the condensation products of the amino acids, the anhydrides, and of other combinations of the amino acids.

Condensation products of amino acids have been obtained by various investigators by simply heating the amino acids to a temperature of about 200 degrees C.

Leucinimide was prepared and studied by Hesse in 1857,[174] by Hesse and Limpricht,[175] and by Kohler.[176] Lactimide was made by Preu[177] from alanine. Anhydrides of aspartic acid were made by Schaal,[178] by

[174] Hesse, O., *J. prakt. Chem.*, 1857, lxx, 34.
[175] Hesse, O., and Limpricht, H., *Ann. Chem.*, 1850, cxvi, 201.
[176] Kohler, A., *Ann. Chem.*, 1865, cxxxiv, 367.
[177] Preu, J., *Ann. Chem.*, 1865, cxxxiv, 372.
[178] Schaal, E., *Ann. Chem.*, 1871, clvii, 24.

Guareschi,[179] and by Grimaux,[180] and the composition of these anhydrides was studied especially by Schiff [181] in several publications appearing between 1897 and 1899. An anhydride of phenylglycine was examined by Meyer [182] in 1877 and phenyllactimide was prepared in 1883 by Erlenmeyer and Lipp [183] from phenylalanine. An anhydride of sarcosine (methylglycine) was carefully studied by Mylius [184] and of all the anhydrides just mentioned this was the only one whose constitution was determined.

In addition to the experiments of Grimaux, who, in 1882, had obtained substances resembling proteins in properties, Schützenberger [185] between 1888 and 1891 carried out some of the first and most successful attempts to synthesize protein-like substances. Recognizing that the decomposition of proteins into amino acids is essentially a phenomenon of hydrolysis, he regarded dehydration as an essential feature of any attempt at protein synthesis, while the abundance of amino acids among the products of protein hydrolysis, and the presence therein, as he thought, of bodies related to urea, led him to believe that protein synthesis must consist in the linkage of amino acids with molecules of urea and the elimination of water. Accordingly amino acids were mixed with urea and phosphorus pentoxide and heated to 125° C. The product was a pasty solid, soluble in water and readily coagulated by alcohol. It was furthermore precipitated from aqueous solutions by the usual protein precipitants and gave the biuret and xanthoproteic reactions.

The experiments of Schützenberger, just as those of Grimaux, threw little light upon the structure of the protein molecule beyond emphasizing the already sufficiently evident probability that the acid amide grouping plays an important part in the building up of the protein molecule.

The clue which led, through a series of remarkable researches, to our present comparatively extensive knowledge of the groupings within the protein molecule, was obtained in 1883 by Curtius [186] when he discovered that ethyl glycocollate (the ethyl ester of glycine) in aqueous solution tends to form glycine anhydride. Researches by Curtius and his co-workers [187] showed that glycine anhydride was probably represented by the structure:

[179] Guareschi, J., *Gazz. chim. ital.*, 1876, vi, 370.
[180] Grimaux, E., *Bull. soc. chim.*, 1882 (2), xxxviii, 64.
[181] Schiff, H., *Ber. chem. Ges.*, 1897, xxx, 2449; *Ann. Chem.*, 1898, cciii, 183; 1899, ccvii, 231; 1900, cccx, 301.
[182] Meyer, P. J., *Ber. chem. Ges.*, 1877, x, 1967.
[183] Erlenmeyer, E., and Lipp, A., *Ann. Chem.*, 1883, ccxix, 179.
[184] Mylius, F., *Ber. chem. Ges.*, 1884, xvii, 286.
[185] Schützenberger, P., *Compt. rend. Acad.*, 1888, cvi, 1407; 1891, cxii, 198.
[186] Curtius, T., *Ber. chem. Ges.*, 1883, xvi, 753.
[187] Curtius, T., and Goebel, F., *J. prakt. Chem.*, 1888 [N.S.], xxxvii, 150; Curtius, T., and Schulz, H., *Ber. chem. Ges.*, 1890, xxiii, 3041.

$$O{=}C\begin{<}{}^{CH_2{-}NH}_{NH{-}CH_2}\end{>}C{=}O$$

which was ultimately proved by Fischer and Fourneau in 1901.[188]

Obviously, if the closed ring representing the glycine anhydride molecule could be opened up without destroying the stability of the molecule, a new amino acid would be formed, one degree more complex than the original amino acid (glycine). This possibility was realized by Emil Fischer (see below).

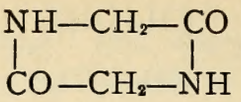
Between the years of 1881 and 1910 Curtius and his pupils published a long series of papers concerning the preparation and properties of complex compounds of certain amino acids and the benzoyl group. Their mode of synthesis was peculiar and they undergo many interesting reactions, all of which are reviewed by Plimmer,[173] but no great interest seems to have been attracted to them.

Synthesis of Polypeptides. Our knowledge of the structure of the protein molecule is due to the systematic researches of Emil Fischer and his pupils which were begun in 1901. To them also is due the proof of the accuracy of Hofmeister's view that the acid amide form of combination of the amino acids is the principal one in the protein molecule. Fischer has termed the combinations of the amino acids which he has prepared, polypeptides, in imitation of the nomenclature adopted for carbohydrates, where we differentiate between mono-, di-, tri-, and poly-saccharides according to the number of units the molecule contains.

The three methods which have been devised by Fischer for the synthesis of the polypeptides are described below. A description of all the polypeptides which have been prepared is impossible, but tables are presented giving the compounds obtained by the various methods. The tables have been taken mainly from Plimmer's Monograph, but have been added to by the authors. Reference should be made to this Monograph and especially to the original articles collected in the two volumes by Emil Fischer on the "Untersuchungen Über Aminosäuren, Polypeptide und Proteine" I and II,[189] for the proper comprehension of the enormous work which has been expended on the synthesis of the polypeptides.

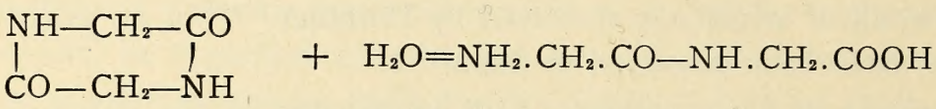
Method I. By formation and hydrolysis of the anhydrides (diketopiperazines). It has been found by previous investigators that leucine, alanine and other amino acids were converted on heating into anhydrides. Glycine, for example, gave the anhydride,

[188] Fischer, E., and Fourneau, E., *Ber. chem. Ges.*, 1901, xxxiv, 2868.
[189] Fischer, E., "Untersuchungen Über Ammosäuren, Polypeptide und Proteine," 1899-1906 (Julius Springer, Berlin, 1906); Vol. II, 1907-1919 (Julius Springer, Berlin, 1923).

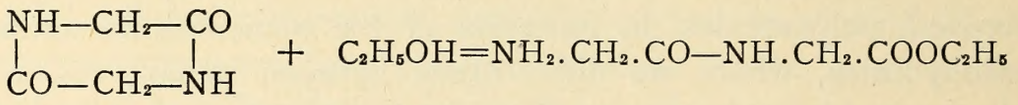


These anhydrides form the starting point in the synthesis of the polypeptides by this method, and they are best obtained by heating the ester of the amino acids in a sealed tube to 150° to 180° C. for some hours.

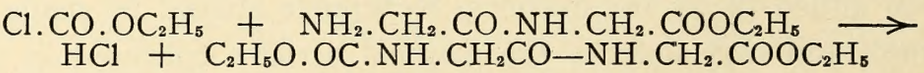
Fischer and Fourneau [188] in 1901 found that glycine anhydride was converted by boiling with concentrated hydrochloric acid into the hydrochloride of an amino acid of the formula $C_4H_8N_2O_3$; from this they obtained the free acid by treatment with the calculated amount of caustic soda, or by means of silver oxide, and represented its formation by the equation:



The new compound is the first anhydride of glycine, and it was termed glycyl-glycine, the group $NH_2.CH_2.CO$ being called the glycyl group. If, instead of treating with hydrochloric acid, the glycine anhydride is treated with alcoholic hydrochloric acid, the ethyl ester of glycyl-glycine is obtained.

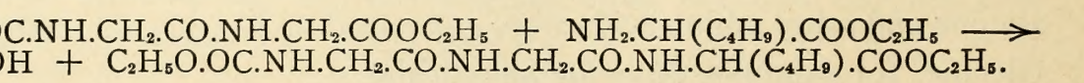


Both the free acid and its ester have a great tendency to become reconverted into glycine anhydride, and both compounds are characterised by the great reactiveness of the NH_2 group; thus with phenyl-isocyanate they both yield the compound, $C_6H_5.NH.CO.NH.CH_2.CO.NH.CH_2.COOH$, and the ester on combination with ethyl chlorocarbonate gives carbethoxyl-glycyl-glycine ester:



from which the amide, $C_2H_5O.OC.NH.CH_2.CO—NH.CH_2.CONH_2$, is obtained by the action of ammonia, and the free acid, $C_2H_5O.OC.NH.CH_2.CO—NH.CH_2.COOH$, by careful hydrolysis with soda.

When carbethoxyl-glycyl-glycine ester was heated with leucine ester combination occurred and the compound, carbethoxyl-glycyl-glycyl-leucine ester, was formed:



This compound contains three amino acids combined together and was the first known representative of a tripeptide.

It would appear, therefore, as if we had only to repeat this cycle of operations indefinitely in order to secure the most complex poly-amino-acids; but this is not so easy as it might appear, since the instability of polyamino acids consequent upon the high reactivity of the amino group, and the consequent difficulty of obtaining simple anhydrides renders this procedure impossible. Moreover, the anhydride ring is in many cases very difficult to break up when it has once been formed, *e.g.*, leucine anhydride.

While this method does not lend itself to the preparation of higher polypeptides, it will be observed that pentaglycyl-glycine and another compound, probably octaglycine anhydride, have been prepared by heating the methyl ester of diglycyl-glycine. Neither is the method applicable to the preparation of mixed anhydrides, as, for example, glycyl-alanine anhydride, since they cannot be obtained by heating a mixture of the esters, for a complex mixture would result, but they are easily prepared by the action of ammonia upon the esters of the dipeptides obtained by the other methods.

The compounds which have been prepared by this method to 1913 are tabulated by Plimmer. Some of the simple polypeptides which have been prepared by this method are: glycyl-glycine, alanyl-alanine, leucyl-leucine, histidyl-histidine, lysyl-lysine, seryl-serine, isoseryl-isoserine, and tyrosyl-tyrosine. Some of the mixed polypeptides are: glycyl-alanine, glycyl-*l*-tyrosine, *l*-tyrosyl-glycine, leucyl-glycine, glycyl-leucine, leucyl-alanine, and alanyl-leucine.

Method II. By means of the halogen acyl compounds. In the search for methods of overcoming the difficulties encountered in the first method of preparing polypeptides Fischer found that the instability of the free amino acids could be eliminated by the introduction of radicals into the amino group. It also occurred to Fischer that the radical thus introduced into the amino group might itself be made to serve as a carrier of amino acid groups into the molecule. This anticipation proved to be correct. Fischer and Otto [190] first described this method of synthesizing polypeptides in 1903. Just as an ordinary acyl radical can be combined with an amino acid, as, for example, in the preparation of benzoylalanine, so also can a halogen substituted acyl radical be combined with an amino acid. The subsequent action of ammonia upon this compound replaces the halogen atom by the amino group and a dipeptide results, thus:

Chloracetyl chloride and alanine yield chloracetyl alanine,

$$ClCH_2.COCl + NH_2.CH(CH_3).COOH \longrightarrow Cl.CH_2.CO.NH.CH(CH_2).COOH + HCl$$

[190] Fischer, E., and Otto, E., *Ber. chem. Ges.*, 1903, xxxvi, 2106, 2993.

from which, by the action of ammonia, glycyl-alanine is obtained:

$$Cl.CH_2.CO.NH.CH(CH_3).COOH + 2NH_3 \longrightarrow NH_2.CH_2.CO.NH.CH(CH_3).COOH + NH_4Cl.$$

In other words, the chloracetyl-group, introduced to protect the amino group of the amino acid, is, after it has performed its protective function, itself transformed into an amino acid group, through the replacement of the halogen atom by $—NH_2$. Obviously, other halogen-containing acid groups may be used in place of chloracetyl, and in this way a great variety of amino acid groups can be introduced into the amino group. Among others the following are employed:

Chloracetyl-chloride for the introduction of the glycyl radical.

α-bromopropionyl-chloride for the introduction of the alanyl radical.

α-bromobutyryl-chloride for the introduction of the α-aminobutyryl radical.

α-bromisocapronyl-chloride for the introduction of the leucyl radical.

Phenyl-bromopropionyl-chloride for the introduction of the phenyl-alanyl radical.

By this method the chain of amino acids is lengthened at the amino group end. In practice this reaction can be carried out in two ways:

1. By the action of the halogen acyl chloride upon the alkaline solution of the amino acid. This reaction proceeds well with the higher acyl chlorides which are not rapidly acted upon by water, but with the lower acyl chlorides it must be carried out at a very low temperature, and the yields even then are in many cases very poor.

2. By the action of the halogen acyl chloride upon the ester of the amino acid in anhydrous solvents, such as ether, chloroform, petroleum ether. In this reaction two molecules of amino acid are required for one molecule of halogen acyl chloride, since half the ester is removed from the reaction as ester hydrochloride. In order to prevent this, the reaction may be carried out in the presence of alkali or alkali carbonate. Subsequent saponification of the ester follows this operation, and loss results by the action of alkali on the halogen acyl radical. This method is only used when the reaction gives bad yields in aqueous solution.

Not only can the acyl radicals be combined with amino acids, but also with di-, tripeptides, etc., thus, theoretically, making as long a chain as desired. This method, however, allows the chain to be lengthened only from the amino group end.

The majority of the polypeptides synthesized by this method are optically inactive, but the optically active compounds can also be prepared by employing the optically active halogen acyl chloride. Since these compounds undergo the Walden inversion, the method allows the whole of

an inactive amino acid being employed for the synthesis of an optically active polypeptide. Thus, *dl*-leucine after separation into *d*-leucine and *l*-leucine can be converted into *l*-leucyl-*l*-leucine by preparing the *d*-bromisocapronyl chloride from the *d*-leucine and combining it with *l*-leucine; treatment with ammonia gives *l*-leucyl-*l*-leucine as the compound undergoes the Walden inversion. The four isomers

d-leucyl-*l*-leucine *l*-leucyl-*d*-leucine	*d*-leucyl-*d*-leucine *l*-leucyl-*l*-leucine
A	*B*

can thus be prepared. The *A* compound is the former inactive leucyl-leucine.

Some of the optically inactive dipeptides prepared by this method are: glycyl-alanine, glycyl-phenylalanine, glycyl-leucine, glycyl-isoleucine, glycyl-glutamic acid, alanyl-glycine, alanyl-alanine, alanyl-phenylalanine, alanyl-serine, valyl-glycine, valyl-alanine, leucyl-glycine, leucyl-alanine, leucyl-aspartic acid, phenylalanyl-alanine, phenylalanyl-leucine, and prolyl-alanine.

Some of the optically active dipeptides prepared by the second method are: glycyl-*d*-alanine, glycyl-*d*-valine, glycyl-*l*-leucine, *l*-alanyl-glycine, *d*-alanyl-*d*-alanine, *d*-alanyl-*d*-valine, *d*-alanyl-*l*-leucine, *d*-alanyl-*l*-tyrosine, *l*-valyl-*d*-valine, *d*-valyl-glycine, *l*-leucyl-glycine, *l*-leucyl-*d*-alanine, *l*-leucyl-*d*-valine, *d*-leucyl-*l*-leucine, *d*-leucyl-*d*-leucine, *l*-leucyl-*l*-histidine, *l*-leucyl-*l*-tryptophane, and *d*-isoleucyl-glycine.

As an illustration of the tripeptides prepared by this method the following may be mentioned: diglycyl-glycine, diglycyl-cystine, glycyl-*d*-alanyl-*l*-tyrosine, *l*-leucyl-glycyl-*l*-aspartic acid, *l*-leucyl-glycyl-*d*-isoleucine, and *d*-alanyl-*l*-leucyl-*d*-isoleucine.

Triglycyl-glycine, *d*-alanyl-diglycyl-glycine, glycyl-*d*-alanyl-*l*-leucyl-*d*-isoleucine, and glycyl-*l*-leucyl-glycyl-*l*-leucine may be mentioned as examples of tetrapeptides and tetraglycyl-glycine and *l*-leucyl-glycyl-*l*-leucyl-glycyl-*l*-leucine are pentapeptides which have been prepared by this method.

Method III. By means of the acid chlorides of the amino acids and of the polypeptides. This is the simplest method of combining together two or more amino acids and differs from the previous one, in that the chain of amino acids can be lengthened at the carboxyl, instead of the amino, end of the molecule. It could not be employed at the commencement of Emil Fischer's researches, since the acid chlorides of the amino acids were unknown. It is now of the greatest importance as it admits of the preparation of any conceivable polypeptide, and it has also given us the knowledge of the most complex compound known by synthesis.

Previous to 1904 it had been impossible to prepare the acid chlorides of the amino acids because of the reactivity of the amino group. In that year Fischer found that if the amino group was rendered more stable by the presence of a halogen acyl group the acid chlorides of the amino acids could be formed by the action of phosphorus pentachloride. The reaction was simply carried out by treatment with phosphorus pentachloride in the presence of acetyl chloride. The acid chlorides thus prepared react with the esters of other amino acids or polypeptides to form chains of greater length. For example:

Bromisocapronylglycine was converted into its acid chloride and combined with glycine ester, when it yielded bromisocapronyl-glycyl-glycine ester,

$$C_4H_9CHBr.CO.NH.CH_2.COCl + 2NH_2.CH_2.COOC_2H_5 \longrightarrow HCl.NH_2.CH_2.COOC_2H_5 + C_4H_9.CHBr.CO.NH.CH_2.CO.NH.CH_2.COOC_2H_5$$

which, on subsequent saponification and treatment with ammonia, gave the tri-peptide leucyl-glycyl-glycine, $C_4H_9.CH(NH_2).CO.NH.CH_2.CO.NH.CH_2.COOH$.

Not only was it possible to prepare the acid chloride of a halogen acyl derivative of an amino acid, but also that of a di-, tri-, etc., peptide by exactly the same means. Thus, the compound bromisocapronyl-diglycyl-glycyl chloride can be obtained, and by condensing it with the esters of amino acids and of polypeptides Fischer has prepared a hexa-, a hepta-, and a deca-peptide.

By continuing the process of preparing the acid chloride of a new polypeptide and again combining it with a polypeptide ester, the synthesis of the complex octadecapeptide, composed of fifteen glycine residues and three leucine residues, was effected.[191] Its preparation is the best illustration of how this method lends itself to the synthesis of the polypeptides. This octadecapeptide has the highest molecular weight of any compound yet prepared by synthesis and of which we know the constitution. Its molecular weight is 1213, a figure which far exceeds that of the fats, tristearin having a molecular weight of only 891.

By applying this method of preparing the acid chlorides to the amino acids themselves, Fischer ultimately succeeded in obtaining the amino acid chlorides, so that he was enabled to combine together any two amino acids in any order, without the necessity of preparing the corresponding halogen derivative. Polypeptides containing the natural optically active amino acids can thus be synthesized with ease, since the natural compound obtained by hydrolysis can be again used directly in

[191] Fischer, E., *Ber. chem. Ges.*, 1907, xl, 1754.

the synthesis, and very often it is easier to prepare the natural compound than the synthetical one, which also requires separation into its stereo-isomers.

This method cannot be used directly for the introduction of tyrosine and other hydroxyamino acids on account of the reactivity of the hydroxyl group with phosphorus pentachloride. If, however, the hydroxyl group be protected by combination with the carbomethoxyl group, the acid chloride can be prepared and used for synthesis.

This method is very useful in the synthesis of those polypeptides containing amino acids, the halogen derivatives of which are not readily obtainable, *e.g.,* tryptophane and proline. Following are some examples of polypeptides synthesized by Method III:

Dipeptides: *d*-alanyl-glycine, *d*-alanyl-*d*-alanine, *dl*-valyl-glycine, *l*-leucyl-glycine, *l*-leucyl-*d*-alanine, *l*-leucyl-*l*-leucine, *l*-prolyl-*l*-phenylalanine, *l*-prolyl-*d*-phenyl-alanine, *d*-tryptophyl-glycine, and *l*-tryptophyl-*d*-glutamic acid.
Tripeptides: leucyl-glycyl-glycine, l-leucyl-*l*-tryptophyl-*d*-glutamic acid, *l*-leucyl-glycyl-*l*-leucine, and glycyl-tyrosyl-glycine.
Tetrapeptides: leucyl-diglycyl-glycine, *d*-alanyl-glycyl-*l*-tyrosine, glycyl-tyrosyl-glycyl-alanine, and glycyl-glutamyl-diglycine.
Pentapeptides: leucyl-triglycyl-glycine and *l*-leucyl-triglycyl-*l*-leucine.
Hexapeptide: leucyl-tetraglycyl-glycine.
Heptapeptide: leucyl-pentaglycyl-glycine.
Octapeptide: leucyl-hexaglycyl-glycine.
Decapeptide: leucyl-octaglycyl-glycine.
Dodecapeptide: leucyl-decaglycyl-glycine.
Tetradecapeptide: leucyl-triglycyl-leucyl-octaglycyl-glycine.
Octadecapeptide: leucyl-trigylcyl-leucyl-trigylcyl-leucyl-octaglycyl-glycine.

For a more complete review of the literature and a more comprehensive treatment of the synthesis and properties of the polypeptides, see Plimmer's Monograph.

CHAPTER II

THE DETERMINATION OF AMINO ACIDS IN PROTEINS

To differentiate properly between the protein bodies it is necessary to know their constitution. A knowledge of constitution, however, is dependent upon a knowledge of composition, and the composition of proteins in terms of the constituent amino acids is a problem that is still far from solution. The bases upon which proteins are classified, *i.e.*, origin, solubility, coagulability on heating, and other physical properties, give but little information concerning the chemical structure of the molecules characteristic of those proteins. Precipitation reactions are too general and elementary analysis does not, except in the case of those proteins containing phosphorus or iron, aid in distinguishing between the proteins, because the native proteins are so nearly alike in these respects. Many of the color reactions, it is true, indicate the presence of certain groups or complexes, but most of these reactions are characteristic of the protein bodies in general, and in but few cases distinguish between individual proteins. Furthermore, these reactions give no quantitative measure of the different complexes which cause them, although these quantitative relations are the most important means of differentiating protein.

Until recently, knowledge regarding the structure of the protein molecule has been chiefly obtained by detailed study of the decomposition products resulting from boiling the protein with strong acids. Proteins, when boiled with acid, are resolved into a mixture of α-amino acids, of which nineteen are now definitely known, and ammonia, together with such non-protein prosthetic groups that may have been present in the molecule. The complete separation of these substances was practically a hopeless undertaking until Emil Fischer [1] in 1901 introduced the method of fractional distillation of the ethyl esters of the amino acids under very low pressure. Since then the knowledge of the amino acid make-up of proteins has grown rapidly, so that by 1918 the composition of from 65 to 85 per cent of the molecule of a number of proteins was known. The technical difficulties were so great, however, that a higher summation of the individual amino acids could hardly be expected.

[1] Fischer, E., *Z. physiol. Chem.*, 1901, xxxiii, 151.

Osborne, Leavenworth and Brautlecht [2] in 1908 proved that the methods for estimating the ammonia content and the diamino acid (the three hexone bases) content of a protein are quite satisfactory, so that we can differentiate proteins with respect to these four products. The complete analysis of a protein, however, is still an impossibility due to the unsatisfactory methods for estimating the several monoamino acids. Even if a complete analysis were possible, a complete knowledge of protein constitution would be a long way off.

In 1918 Dakin [3] showed that the monoamino acids could be separated as a group from the hydrolysis mixture by long-continued extraction with butyl alcohol, in a form eminently suitable for esterification and subsequent separation by Fischer's method. Dakin's procedure is probably the most important advance in the technic of protein analysis of recent years, and has enabled him to increase the summation of amino acids in gelatin to 91.3 per cent,[4] and in zein, the most abundant protein in corn, to 100 per cent.[5] These figures are unprecedented and, as Vickery [6] in 1924 stated, "It seems highly improbable that any appreciable amount of a hitherto unknown amino acid can occur in zein, and the day does not seem far distant when we shall know the composition of the whole of the molecule of at least one protein."

HYDROLYSIS

Hydrolysis by acids, alkalies, or proteolytic enzymes has proved to be the best means of investigating the composition of the protein molecule. In 1820 Braconnot hydrolyzed gelatin by boiling with dilute sulfuric acid. Between 1850 and 1875 Ritthausen, Hlasiwetz and Habermann, and others favored hydrochloric acid as a hydrolyzing agent, and Schutzenberger, between 1870 and 1880, used baryta water under pressure. The action of vegetable enzymes on proteins has been studied especially by Schulze and his co-workers, while the action of animal enzymes has been extensively studied by Kühne, Kossel, Kutscher, Drechsel, and others.

Hydrolysis with Hydrochloric Acid

Hydrochloric acid has been a popular reagent in the hydrolysis of proteins. Hlasiwetz and Habermann [7, 8] carried out the hydrolysis in the presence of stannous chloride so that the solution, which usually

[2] Osborne, T. B., Leavenworth, C. S., and Brautlecht, C. A., *Am. J. Physiol.*, 1908, xxiii, 180.
[3] Dakin, H. D., *Biochem. J.*, 1918, xii, 290.
[4] Dakin, H. D., *J. Biol. Chem.*, 1920, xliv, 499.
[5] Dakin H. D., *Z. physiol. Chem.*, 1923, cxxx, 159.
[6] Vickery, H. B., *J. Ind. Eng. Chem.*, 1924, xvi, 1029.
[7] Hlasiwetz, H., and Habermann, J., *Ann. Chem.*, 1871, clix, 304.
[8] Hlasiwetz, H., and Habermann, J., *Ann. Chem.*, 1873, clxix, 150.

becomes dark brown, would remain colorless. Fischer and others, however, did not consider the addition necessary, although they used fairly strong acid. The hydrolysis of protein with strong hydrochloric acid is usually carried out by boiling the protein with three times its quantity of concentrated hydrochloric acid (sp. gr. 1.19). The length of time necessary to effect complete hydrolysis varies usually from six to twenty-four hours, depending on the particular protein.

Van Slyke [9] showed that proteins may be hydrolyzed equally well by boiling them with from 10 to 20 parts of 20 per cent hydrochloric acid for 15 to 18 hours. Henriques and Gjaldbäk [10] and Van Slyke [11] have found that complete hydrolysis is produced by heating the protein with 3*N* hydrochloric acid in an autoclave. The former investigators do not state the quantity of acid used, but Van Slyke used thirty-three times more acid than protein. Van Slyke showed further that complete hydrolysis was effected either by heating at 100° for 48 hours with 20 per cent acid, or at 150° with 3*N* acid. Henriques and Gjaldbäk [10] found that the hydrolysis of egg albumin was not complete after heating for 12 hours with concentrated acid, but was complete on heating at 150° for 1.5 hours in an autoclave with 3*N* acid. They found no advantage in heating at 150° for a longer time; at 180° in an autoclave decomposition of the amino acids occurred with the formation of ammonia. Finally, Zelinsky and Ssadikow [12] have recently shown that complete hydrolysis may be effected with very weak acids. They state that proteins may be completely hydrolyzed by heating them with 4, 3, 2, 1, or even 0.5 per cent hydrochloric acid in an autoclave at 180° for three to six hours. Dowell and Menaul [13] completely hydrolyzed various proteins by heating with 10 per cent hydrochloric acid in an autoclave for three hours at a pressure of 20 pounds.

Pfannl [14] and Pribram [15] have been able to hydrolyze proteins by boiling with alcohol saturated with hydrogen chloride. While hydrolysis occurred with carefully dried proteins and carefully dried reagents, it was more complete if the alcohol contained from 3 to 5 per cent water. Abderhalden and Hanslian [16] obtained no appreciable hydrolysis when moisture was carefully excluded during all operations. Weizmann and Agashe [17] have isolated small quantities of amino acids from such hydrolysates, but consider that the main result of hydrolysis with alco-

[9] Van Slyke, D. D., *J. Biol. Chem.*, 1911, x, 15.
[10] Henriques, V., and Gjaldbäk, J. K., *Z. physiol. Chem.*, 1910, lxvii, 8.
[11] Van Slyke, D. D., *J. Biol. Chem.*, 1912, xii, 295.
[12] Zelinsky, N., and Ssadikow, W., *Biochem. Z.*, 1923, cxxxviii, 156.
[13] Dowell, C. T., and Menaul, P., *J. Biol. Chem.*, 1919, xl, 131.
[14] Pfannl, M., *Monatsh. Chem.*, 1910, xxxi, 81.
[15] Pribram, B. O., *Z. physiol. Chem.*, 1911, lxxi, 472.
[16] Abderhalden, E., and Hanslian, R., *Z. physiol. Chem.*, 1912, lxxvii, 285.
[17] Weizmann, C., and Agashe, G. S., *Biochem. J.*, 1913, vii, 437.

holic hydrochloric acid is the formation of complex products intermediate between protein and amino acids. Herzig and Landsteiner [18] found that hydrolysis with alcoholic hydrochloric acid took place only to a small extent.

Hydrolysis with Sulfuric Acid

While hydrochloric acid is, perhaps, more often used as a hydrolytic agent for proteins, sulfuric acid is also a common reagent. Braconnot [19] in 1820 hydrolyzed gelatin by boiling with dilute sulfuric acid. In the hydrolysis of protein material with sulfuric acid the protein is usually boiled for 15 to 24 hours with six times its quantity of 25 to 33 per cent sulfuric acid, or with a mixture of three times its weight of concentrated sulfuric acid and six times its weight of water. Generally, the mixture is heated under a reflux condenser, first on a steam-bath for an hour or two or until frothing has ceased, and then in an oil bath at about 105° until hydrolysis is complete. Dakin [4] effected hydrolysis of 250 grams of gelatin by first boiling for 12 to 20 hours with a mixture of 300 cc. of concentrated sulfuric acid and 650 cc. of water, and then heating for 8 hours in an autoclave at 135 to 140°. The main advantage for hydrolysis with sulfuric acid rather than hydrochloric acid is that the acid may be readily removed from the hydrolysate by precipitation as barium sulfate.

Comparison of the Hydrolysis with Hydrochloric and Sulfuric Acids

In an experiment by Kossel and Kutscher [20] three samples of casein were hydrolyzed as follows: (*a*) by sulfuric acid for 12 hours, (*b*) by concentrated hydrochloric acid for 12 hours, and (*c*) by dilute (1:2) hydrochloric acid for 3 hours. From the results quoted below it was decided to use the dilute hydrochloric acid for hydrolysis:

	a	*b*	*c*
	(*per cent*)	(*per cent*)	(*per cent*)
Histidine	0.9	0.76	1.56
Arginine	4.45	4.21	4.50
Lysine	1.84	2.29	2.0

Other investigators have claimed that a difference was obtained by hydrolyzing with concentrated hydrochloric acid and with 25 to 33 per cent sulfuric acid, but experiments by Abderhalden and Funk [21] and

[18] Herzig, J., and Landsteiner, K., *Biochem. Z.*, 1914, lxvii, 334.
[19] Braconnot, H., *Ann. chim. phys.*, 1820, xiii, 113.
[20] Kossel, A., and Kutscher, F., *Z. physiol. Chem.*, 1900, xxxi, 165.
[21] Abderhalden, E., and Funk, C., *Z. physiol. Chem.*, 1907, liii, 19.

by Skraup and Türk [22] have proved that complete hydrolysis is effected by both reagents, provided boiling is continued for a sufficient length of time.

Hydrolysis with Other Acids

Hugounenq and Morel [23] have made a special study of the hydrolysis of proteins with hydrofluoric acid and found that the results depend upon the strength of the acid: the stronger the acid the greater is the amount of complex polypeptides. In their experience it required many hours' boiling with dilute acid to bring about complete hydrolysis.

Zelinsky and Ssadikow,[12] who made a study of hydrolysis with dilute acids, report that materials such as horn, feathers, collagen, casein, horse blood fibrin, etc., are completely hydrolyzed, as indicated by a negative biuret test, by heating in an autoclave at 180° with a 10 per cent solution of formic acid. Egg albumin was found to be very resistant; six hours' heating left some insoluble material and the solution still showed a biuret reaction. A 50 per cent solution of formic acid, however, completely hydrolyzed this substance at 180°. Acetic acid can also be used; for example, for the hydrolysis of gelatin but six hours' heating in the autoclave are required with this acid, whereas three hours is sufficient with formic acid. According to these authors, hydrolysis in the autoclave with dilute acids has the great advantage that the hydrolysates obtained are completely clear or only slightly yellow in color, and that the formation of humin substances, which always occurs when concentrated acids are used, does not occur.

Comparison of the Hydrolysis with Acids and Alkalies

While proteins are readily hydrolyzed by boiling with alkalies, alkali hydrolysis is not general and is only used for special purposes. The amino acids produced by alkali hydrolysis are completely racemized, whereas in acid hydrolysis only partial racemization takes place. Arginine is split by boiling with alkali into ornithine and ammonia and cystine is also decomposed. Abderhalden, Medigreceanu, and Pincussohn [24] compared the hydrolysis by acids and by alkalies and found that alkalies produce the most complete hydrolysis. Abderhalden and Brahm [25] found that alkalies were the more effective hydrolytic agents; a substance formed from silk, and resistant to acid hydrolysis, was completely hydrolyzed by alkali.

[22] Skraup, Zd. H., and Türk, W., *Monatsh. Chem.*, 1909, xxx, 287.
[23] Hugounenq, L., and Morel, A., *Compt. rend. Acad. Sci.*, 1908, cxlvi, 1291; *Bull. Soc. chim.*, 1908 (4), iii, 1146; *J. Pharm. Chim.*, 1908 (6), xxviii, 486; *Compt. rend. Acad. Sci.*, 1909, cxlviii, 236; cxlix, 41.
[24] Abderhalden, E., Medigreceanu, F., and Pincussohn, L., *Z. physiol. Chem.*, 1909, lxi, 205.
[25] Abderhalden, E., and Brahm, C., *Z. physiol. Chem.*, 1909, lxi, 256.

Hydrolysis with Enzymes

The hydrolysis of proteins by the proteolytic enzymes occurring in animals and in plants has often been investigated. Schulze and his co-workers have made a special study of hydrolysis by means of plant enzymes, while Kühne, Kossel, Kutscher, Drechsel and others have studied the action of animal enzymes on protein material. Hydrolysis by means of proteolytic enzymes, however, is never complete and early investigators noted that a complex substance which was resistant to further action of trypsin was usually formed. Fischer and Abderhalden [26] have shown that this resistant substance contains all the phenylalanine and proline present in the protein molecule. Even under the combined action of pepsin and trypsin, although phenylalanine and proline are formed, a substance still resistant to trypsin hydrolysis remains. Almost complete hydrolysis may be effected by the combined action of trypsin and the enzymes of the small intestine if digestion is allowed to continue long enough. Henriques and Gjaldbäk [10] found that even after prolonged digestion from 5 to 10 per cent of the total amino acids still remained in combination.

Hydrolysis by enzymes has been of greatest service in the examination of proteins for their various amino acids, since the treatment is less likely to destroy or change these units themselves. Hydrolysis by this method is not serviceable for a complete analysis of the decomposition products of the protein molecule.

Determination of the Completeness of Hydrolysis

The completeness of hydrolysis has usually been determined by performing the biuret test on the solution and on any residue which may be left. If either gives a positive test hydrolysis is continued until neither the solution nor the residue gives the test. Many proteins are hydrolyzed very slowly no matter what the hydrolytic agent may be. Osborne and Guest [27] found, in the case of casein, that hydrolysis with concentrated hydrochloric acid required from three to five days. As has been pointed out by Osborne and Jones,[28] the biuret test alone is not sufficient in many cases, as it is necessary to boil with acids for a longer time than is indicated by this negative test.

The biuret test, therefore, is not a satisfactory test to indicate completeness of hydrolysis. A better indication of completeness of hydrolysis is to determine whether an increase of ammonia and amino nitrogen

[26] Fischer, E., and Abderhalden, E., *Z. physiol. Chem.*, 1903, xxxix, 81; 1903, xl, 215.
[27] Osborne, T. B., and Guest, H. H., *J. Biol. Chem.*, 1911, ix, 333.
[28] Osborne, T. B., and Jones, D. B., *Am. J. Physiol.*. 1910, xxvi, 305.

occurs in a subsequent period of boiling. Such an increase may be determined by following the course of hydrolysis with the Van Slyke amino nitrogen method or by Sörensen's method of titration with formalin. Another method, which, however, is not as convenient as either of the two just mentioned, is Siegfried's carbamino method. A fourth and very sensitive method is Harding and MacLean's colorimetric method with ninhydrin. This method, however, is inapplicable to strongly acid or alkaline solutions.

Van Slyke's Amino Nitrogen Method. Perhaps the most accurate, and at the same time a very convenient method for determining the completeness of hydrolysis of proteins is that of Van Slyke,[9] who describes the method somewhat as follows: The protein is dissolved in 10 to 20 parts of 20 per cent hydrochloric acid, and boiled in a tared flask under a reflux condenser. At intervals of six to eight hours the hydrolysis is stopped, the solution cooled, and 1 to 2 cc. are withdrawn with a pipette. These portions are diluted to 10 cc. and the amino nitrogen determined by liberation with nitrous acid in the Van Slyke apparatus, the reaction being allowed to proceed for five minutes without shaking and then with shaking for one minute. The flask in which the hydrolysis is being carried on is weighed and the solution boiled for another period of six to eight hours. After cooling the flask is again weighed and if loss has occurred water may be added to replace the loss or a correction may be made for the decrease in volume. The hydrolysis is continued until the concentration of amino nitrogen is constant.

Sörensen's Titration Method. Sörensen's method [29] for the estimation of amino acids consists in neutralizing the basicity of the amino groups by coupling with formaldehyde * and then titrating the free carboxyl groups with standard alkali. Sörensen and Jessen-Hansen [30] have modified the original procedure in order to make it applicable to the dark brown solutions resulting from the hydrolysis of proteins, especially by acids. They suggest the precipitation of silver chloride in the solution. The precipitate carries down with it the coloring matter. The amino acid content is then determined by the addition of formalin and subsequent titration with standard alkali in the usual manner. Hydrolysis is continued until there is no increase in amino acid content.

Siegfried's Carbamino Reaction. An interesting, but less convenient, method for following the course of hydrolysis is that of

[29] Sörensen, S. P. L., *Biochem. Z.*, 1907, vii, 45.
* According to Harris this explanation is not entirely correct; see page 134.
[30] Sörensen, S. P. L., and Jessen-Hansen, H., *Biochem. Z.*, 1907, vii, 407.

Siegfried.[31, 32] Siegfried found that, just as ammonia and carbonic acid unite to form ammonium carbonate and ammonium carbamate, the amino acids, through their amino groups, unite with carbonic acid to form carbamino acids, the calcium salts of which are soluble in water. The calcium salts have the following structure:

$$\begin{matrix} R.CH.CO.O \\ | \qquad\quad > Ca. \\ NH.CO.O \end{matrix}$$

On boiling an aqueous solution containing these calcium salts, the salts are decomposed, precipitating calcium carbonate and freeing the amino acids. If one first determines the nitrogen in the aqueous solution containing the calcium salts, then boils to precipitate the calcium carbonate, which is filtered, dried, weighed, and its CO_2 content calculated, a ratio between the number of nitrogen atoms and the molecules of CO_2 which have been in union may be obtained. If the result is expressed as the quotient CO_2/N, the monoamino acids will have a quotient of 1/1 or 1. A quotient of 1 would therefore mean that all the amino acids in the mixture were monoamino acids and that all the amino groups are free, since the reaction only occurs between free amino groups and free carboxyl groups. If diamino acids were present, or if the amino acids were in part combined in peptide form, the quotient would be less than one, since the amount of nitrogen in the solution would be greater than the number of free amino groups. It is possible, therefore, by studying this quotient to follow the course of protein hydrolysis and to determine when hydrolysis is complete.

The Ninhydrin Method. Ruhemann[33] discovered that all acids containing a free amino group in the alpha position reacted with triketohydrindene hydrate (commercially known as ninhydrin) with the production of an intense blue color. Abderhalden and Schmidt[34] have made use of this reaction in the detection of amino acids and it is one of the most sensitive color reactions for this purpose. Harding and MacLean[35] have developed this reaction into a quantitative colorimetric method for estimating the amino acid α-nitrogen and have shown that the method can be used as a means of following protein hydrolysis.[36]

The results obtained by this method are in striking agreement with those obtained by the Van Slyke method, and the general agreement of the two with the Sörensen method show further that the colorimetric

[31] Siegfried, M., *Z. physiol. Chem.*, 1904, xliii, 50; 1905, xliv, 85; 1906, xlvi, 401; 1907, lii, 506.
[32] Siegfried, M., and Neumann, C., *Z. physiol. Chem.*, 1908, liv, 423; Liebermann, H., *ibid.*, 1908, lviii, 84.
[33] Ruhemann, S., *J. Chem. Soc.*, 1910, xcvii, 2025.
[34] Abderhalden, E., and Schmidt, H., *Z. physiol. Chem.*, 1912, lxxxv, 143.
[35] Harding, V. J., and MacLean, R. M., *J. Biol. Chem.*, 1915, xx, 217.
[36] Harding, V. J., and MacLean, R. M., *J. Biol. Chem.*, 1916, xxiv, 503.

method estimates quantitatively the extent of hydrolysis of the peptide chain. The agreement of the colorimetric and gasometric methods is to be expected, for both give theoretical results with aqueous solutions of pure amino acids, except in the case of glycine with the gasometric method, and cystine with both methods.

While the ninhydrin colorimetric method is undoubtedly a most sensitive and also a relatively simple method for determining quantitatively the amino acid content of a protein hydrolysate, it is inapplicable in strongly acid or alkaline solutions. The colorimetric method should find its greatest usefulness in the study of protein hydrolysis in neutral and faintly alkaline media, such as the hydrolysates from enzyme digestion.

HUMIN FORMATION

When proteins are hydrolyzed with acids a dark brown or black precipitate is usually formed. This dark colored precipitate is called humin or melanin and is important on account of its bearing on the quantitative determination of certain of the amino acids. Udránsky [37] and Hoppe-Seyler [38] have shown that humin substances are formed when carbohydrates are boiled with acids, and if nitrogenous material is present, the humins may also contain nitrogen. Mulder [39] was the first to show that albumins separate flocculi of a brown or black color on being boiled with concentrated hydrochloric or sulfuric acids. Hausmann [40] made similar observations in the case of globin. Samuely [41] suggested that the humin formed from proteins was due to secondary reactions between amino acids and carbohydrates, and obtained melanins containing nitrogen by boiling various amino acids with hydrochloric acid in the presence of carbohydrates. Maillard [42] carried out similar experiments and obtained humin-like substances when a number of the amino acids were boiled with various sugars. Alanine was especially reactive and of the sugars xylose and arabinose reacted most rapidly. These humins were shown by Maillard [43] to yield cyclic bases on heating, and he suggested that cellulose and protein were the origin of the pyridine and allied bases found in coal-tar. Pyridine, skatol, pyrrol, and other bases have been prepared from such melanins.[41, 43, 44, 45, 46, 47]

[37] Udránsky, L. V., *Z. physiol. Chem.*, 1888, xii, 33.
[38] Hoppe-Seyler, F., *Z. physiol. Chem.*, 1889, xiii, 66.
[39] Mulder, G. J., in Mann, G., "Chemistry of the Proteids," London, 1906, 87.
[40] Hausmann, W., *Z. physiol. Chem.*, 1900, xxix, 136.
[41] Samuely, F., *Beitr. chem. Phys. Path.*, 1902, ii, 355.
[42] Maillard, L. C., *Compt. rend. Acad. Sci.*, 1913, cliv, 66.
[43] Maillard, L. C., *Compt. rend. Acad. Sci.*, 1913, clvii, 850.
[44] Pictet, A., and Chou, T. Q., *Compt. rend. Acad. Sci.*, 1916, clxii, 127.
[45] Maillard, L. C., *Compt. rend. Acad. Sci.*, 1916, clxii, 757.
[46] Berdez, J., and Nencki, M., *Arch. exp. Path. Pharm.*, 1886, xx, 346.
[47] Nencki, M., and Sieber, N., *Arch. exp. Path. Pharm.*, 1887, xxiv, 17.

Gortner and Blish [48] heated zein, a protein which contains neither tryptophane nor carbohydrate and only a small amount of histidine and which was known to yield but very little humin on hydrolysis, with acid in the presence of tryptophane and carbohydrate and obtained 86.6 per cent of the nitrogen of the tryptophane in the form of humin. With histidine in the place of tryptophane 0.5 per cent of its nitrogen was contained in the humin. Gortner and his collaborators [49, 50] have made an extensive study of the humins which form when a tryptophane-containing protein is boiled with strong acids and have collected evidence which supports the view that the humin is formed by a condensation of the indole nucleus of tryptophane with an unknown aldehydic constituent of the protein molecule.

Tryptophane, therefore, is largely concerned in the formation of humin resulting from acid hydrolysis of proteins. Grindley and Slater,[51] Gortner,[49] Roxas,[52] and Dowell and Menaul [13] have shown that other amino acids may be concerned with humin formation. Roxas [52] found that alanine, leucine, phenyl-alanine and glutamic acid are not factors in humin formation, while proline may be under certain conditions. Tryptophane gave up 71.0 per cent of its nitrogen, tyrosine 15.0, cystine 3.1, arginine 2.33, lysine 2.62, and histidine 1.84. The three hexone bases, arginine, lysine, and histidine, reacted more readily in weak acid solutions than in strong. Arginine, histidine and tryptophane reacted with loss of reactivity of their amino nitrogen towards nitrous acid, but tyrosine and cystine reacted without any such loss.

The work that has been reported on the formation of humins in acid hydrolysis of proteins by various workers [41, 48, 49, 50, 52, 53, 54] gives two general views regarding the nature of humin formation. First it has been shown that humin-like substances may be made by oxidation of pure amino acids and other pure compounds with hydrogen peroxide. On the other hand, there is some evidence that condensation may be the only reaction involved. Between these extremes is the view that the humin of protein hydrolysis is the product of condensation between certain amino acids and an aldehydic group, with or without oxidation.

Roxas [52] advanced the theory that the condensation of sugars with amino acids led to ring formation. Most of the accumulated data, however, point to the conclusion that the artificial humin formed during

[48] Gortner, R. A., and Blish M. J., *J. Am. Chem. Soc.*, 1915, xxxvii, 1630.
[49] Gortner, R. A., *J. Biol. Chem.*, 1916, xxvi, 177.
[50] Gortner, R. A., and Holm, G. E., *J. Am. Chem. Soc.*, 1917, xxxix, 2477; *ibid.*, 1920, xlii, 821; Holm, G. E., and Gortner, R. A., *ibid.*, 1920, xlii, 632; 1920, xlii, 2378; Gortner, R. A., and Norris, E. R., *ibid.*, 1923 xlv, 550.
[51] Grindley, H. S., and Slater, M. E., *J. Am. Chem. Soc.*, 1915, xxxvii, 2762.
[52] Roxas, M. L., *J. Biol. Chem.*, 1916, xxvii, 71.
[53] Fürth, O., and Lieben, F., *Biochem. Z.*, 1920, cix, 124.
[54] Adler, O., and Wiechowski, W., *Ber. chem. Ges.*, 1922, lv, 3, 3030.

the acid hydrolysis of proteins is not a simple condensation product, but is due to a condensation followed by rearrangement or oxidation or both, with the ultimate formation of an extremely resistant molecule or molecules. The nature of the chemical reactions involved and the structural configuration of the humin molecule or molecules formed still require elucidation.

Another interesting case of humin formation is that resulting when tyrosine is oxidized with tyrosinase. Bertrand [55] described this effect as follows: A solution of tyrosine to which an extract of tyrosinase is added first becomes red, then inky black, and finally deposits a black precipitate. Raper [56, 57, 58, 59] and his collaborators have done much to explain the mechanism of the reactions involved and the structure of the intermediate compounds formed in the tyrosinase-tyrosine reaction. The red pigment first formed is probably 5 : 6-quinone of dihydroindole-2-carboxylic acid. Some intramolecular rearrangement takes place with the formation of 5 : 6-dihydroxyindole and 5 : 6-dihydroxyindole-2-carboxylic acid, which are regarded as the immediate precursors of the melanin finally formed.

The formation of humin, therefore, interferes to a considerable extent in the analysis of the protein molecule. Of the total nitrogen in the protein, usually from 1 to 2 per cent is found in the insoluble humin. Another loss is due to the soluble brown humin which colors the solution. Tryptophane especially is lost in the insoluble humin fraction while tyrosine, at least, is concerned in the formation of the soluble humin.

METHODS FOR THE DIFFERENTIATION OF PROTEINS

The Method of Hausmann

Hausmann,[60, 40] working in Hofmeister's laboratory, in 1899 proposed a method for the differentiation of proteins based on the estimation of the nitrogen as distributed in three groups of compounds, *i.e.*, amide nitrogen, diamino nitrogen, and monoamino nitrogen. Previous workers had been isolating the cleavage products and gradually accumulating knowledge of them; but, the methods of isolation not being entirely satisfactory, conclusions as to the entire protein molecule were doubtful. It was a distinct advance when to this information, which, formerly, had been mainly qualitative, there was added the quantitative determination of the amount of the different forms of nitrogen present.

[55] Bertrand, G., *Compt. rend. Soc. Biol.*, 1896, cxxii, 1215.
[56] Raper, H. S., and Wormall, A., *Biochem. J.*, 1923, xvii, 454; 1925, xix, 84.
[57] Raper, H. S., and Speakman, H. B., *Biochem. J.*, 1926, xx, 69.
[58] Raper, H. S., *Biochem. J.*, 1926, xx, 135.
[59] Raper, H. S., *Biochem. J.*, 1927, xxi, 89.
[60] Hausmann, W., *Z. physiol. Chem.*, 1899, xxvii, 95.

Hausmann's method consists in hydrolyzing the protein with strong hydrochloric acid and then determining: (*a*) the nitrogen which can be expelled from the solution as ammonia by distillation with magnesia; (*b*) that which is precipitated by phosphotungstic acid from the solution thus freed from ammonia; and (*c*) the nitrogen remaining in the filtrate from the phosphotungstic acid precipitate. In this way Hausmann thought the amide nitrogen (*a*), the diamino nitrogen (*b*), and the remaining nitrogen (*c*), belonging wholly to monoamino acids, might be quantitatively determined.

Numerous objections to the accuracy of this method have been raised. Henderson [61] maintained that the amount of amide nitrogen varied according to the strength of the acid used and the time of hydrolysis. Schulze and Winterstein [62] found ammonia in the filtrate from the phosphotungstic acid precipitate and Hart [63] obtained more ammonia from the hydrolysate when the distillation was made with magnesia than when made with barium carbonate. Kutscher [64] found that certain of the diamino acid phosphotungstates were appreciably soluble under certain conditions, and claimed, as did also Chittenden and Eustis,[65] that the proportion of the diamino acids precipitated by phosphotungstic acid varied with the conditions under which the precipitation was carried out. Wetzel,[66] also, was unable to obtain consistent results in the phosphotungstic acid precipitation. Schulze and Winterstein [67] next studied the reaction of some of the monoamino acids towards phosphotungstic acid and found that phenylalanine, under certain conditions, was precipitated.

Osborne and Harris [68] especially, and also Gümbel,[69] Rothera,[70] Osborne, Leavenworth and Brautlecht,[2] and Jodidi and Moulton [71] have examined these objections critically. According to Osborne and Harris, Henderson's statement that the amount of ammonia obtained depends on the strength of the acid with which the protein is boiled, as well as on the time of boiling, is doubtless correct, but it was necessary to increase the strength of the acid very considerably or to prolong the boiling in order to cause a real effect on the result. Denis [72] also tested the effect of time of hydrolysis with acid. Casein boiled 70 hours gave

[61] Henderson, Y., *Z. physiol. Chem.*, 1900, xxix, 47.
[62] Schulze, E., and Winterstein, E., *Z. physiol. Chem.*, 1901, xxxiii, 547.
[63] Hart, E., *Z. physiol. Chem.*, 1901, xxxiii, 347.
[64] Kutscher, F., *Z. physiol. Chem.*, 1900, xxxi, 215.
[65] Chittenden, R. H., and Eustis, A. C., *Am. J. Physiol.*, 1900, iii, Proc. XXXI.
[66] Wetzel, G., *Z. physiol. Chem.*, 1900, xxix, 386.
[67] Schulze, E., and Winterstein, E., *Z. physiol. Chem.*, 1901, xxxiii, 574.
[68] Osborne, T. B., and Harris, I. F., *J. Am. Chem. Soc.*, 1903, xxv, 323.
[69] Gümbel, T., *Beitr. chem. Physiol. Path.*, 1904, v, 297.
[70] Rothera, C. H., *Beitr. chem. Physiol. Path.*, 1904, v, 442.
[71] Jodidi, S. L., and Moulton, S. C., *J. Am. Chem. Soc.*, 1919, xli, 1526.
[72] Denis, W., *J. Biol. Chem.*, 1910, viii, 427.

no more ammonia than did the same amount boiled 15 hours. With gelatin there was a slight increase in the amount of ammonia by prolonged boiling.

According to Osborne, Leavenworth, and Brautlecht,[2] ammonia determinations on the same protein hydrolyzed under widely different conditions are practically identical and the amounts of ammonia obtained from a protein hydrolysate by distillation with magnesia at 100° and *in vacuo* at 40° are practically the same. Since none of the amino acids which are known products of protein hydrolysis yield ammonia under these conditions, and since there is such marked agreement between the amounts of ammonia as determined and those calculated to have originated from the dibasic amino acids, it is improbable that the proteins contain any nitrogen other than amide nitrogen which is easily converted into ammonia. Considering all these facts, these investigators claim that ammonia originating from acid hydrolysis of proteins can be determined with very great accuracy, and that this ammonia originates from an amide union in the protein molecule with dibasic acid groupings.

Kutscher lays much stress on the solubility of the phosphotungstic acid precipitate, but Osborne and Harris have shown that, while the volume of the precipitate appears to diminish, it is due to a change in the character of the precipitate and that the actual quantity dissolved is small. This is in agreement with Van Slyke's [9] findings.

The amount of nitrogen precipitated by phosphotungstic acid does vary with the conditions under which the precipitation is carried out. By controlling carefully these conditions, however, Osborne and Harris, Van Slyke, and others have shown that this method may be used with a good degree of accuracy in the separation of the diamino and monoamino acids in a protein hydrolysate.

From the preceding statements, it is evident that Hausmann's method, as he defined it, cannot be used to determine accurately the proportion of amide, diamino, and monoamino nitrogen in a solution containing these protein hydrolytic products. Osborne and Harris,[68] however, have shown that, by careful control of the conditions, it is possible to obtain valuable comparative results, whereby differences between the various proteins are made plainly evident. Much important information concerning the differences between the natural proteins has been obtained by these investigators by the use of this method.

For the differentiation of proteins by the Hausmann method, Plimmer [73, p. 87] gives the details of a procedure which is essentially that of

[78] Plimmer, R. H. A., "The Chemical Constitution of the Proteins," Part I. (London), 1917.

Osborne and Harris [68] combined with Gümbel's [69] method of distilling the ammonia *in vacuo* at 40°. Another improvement incorporated in this procedure is that the original concentration of the hydrolysate, in which most of the hydrochloric acid is removed, is carried out *in vacuo* at 40° instead of on the steam bath.

The Method of Kossel and Kutscher

In 1900 Kossel and Kutscher [20] published their method for the estimation of the three diamino acids—arginine, histidine, and lysine. This method was the first to give approximately accurate quantitative results for these constituents and still appears to be one of the best means available for distinguishing between the several proteins.

The method depends upon the precipitation of arginine and histidine as their silver salts, their separation by difference in solubility in water and in alkaline solutions, and the precipitation of lysine from the filtrate first by phosphotungstic acid, and finally by picric acid. The method was modified in 1903 by Kossel and Patten [74] and by Steudel,[75] in 1906 by Kossel and Pringle,[76] in 1908 by Osborne, Leavenworth, and Brautlecht,[2] and in 1915 Osborne, Van Slyke, Leavenworth, and Vinograd [77] added some noteworthy changes, making the method somewhat more convenient.*

The complete details of the method have been described by Weiss [78] in 1907, by Steudel [79] in 1910, and by Plimmer [73, p. 55] in 1917. The essential steps in the procedure are as follows:

About 25 to 50 grams of protein are hydrolyzed with sulfuric acid and the acid removed with baryta. Ammonia is determined by distilling 100 cc. aliquots with magnesia and the remainder of the solution freed from ammonia by evaporating with magnesia on the steam-bath. Osborne, Leavenworth and Brautlecht [2] concentrate the filtrate from the first barium sulfate precipitate, while still slightly acid, *in vacuo* at about 70° and, after the estimation of the ammonia in an aliquot, the solution is freed from ammonia by evaporation in an open dish on the steam-bath after adding an excess of barium carbonate.

The clear solution, freed from ammonia and containing a small quantity of sulfuric acid, is placed in a large flask and a hot saturated solution of silver sulfate added carefully until a sufficient amount is

[74] Kossel, A., and Patten, A. J., *Z. physiol. Chem.*, 1903, xxxviii, 39.
[75] Steudel, H., *Z. physiol. Chem.*, 1903, xxxvii, 219.
[76] Kossel, A., and Pringle, H., *Z. physiol. Chem.*, 1906, xlix, 318.
[77] Osborne, T. B., Van Slyke, D. D., Leavenworth, C. S., and Vinograd, M., *J. Biol. Chem.*, 1915, xxii, 259.
* The most recent modification is by Vickery and Leavenworth (*J. Biol. Chem.*, 1928, lxxvi, 707). See footnote on page 110 for essential changes.
[78] Weiss, R., *Z. physiol. Chem.*, 1907, lii, 107.
[79] Steudel, H., in Abderhalden, E., "Handbuch der Biochemischen Arbeitsmethoden," Band II, 1910, 498.

present to precipitate all the arginine and histidine. Osborne, Leavenworth and Brautlecht used silver nitrate instead of silver sulfate for this precipitation, because the former is more soluble. The mixture is allowed to cool to 40° and is then saturated with finely powdered baryta. The precipitate, which consists of the silver salts of arginine and histidine, is filtered off and washed with baryta water. The filtrate contains the lysine.

The precipitate of the silver salts of arginine and histidine is suspended in dilute sulfuric acid and decomposed with hydrogen sulfide. The silver sulfide is removed by filtration and the sulfuric acid in the filtrate is then precipitated and filtered off as the barium salt. The solution is concentrated, acidified with nitric acid, and then treated with silver nitrate until a test drop gives a yellow-brown color with baryta. The solution is next exactly neutralized with baryta and 5 cc. of a cold saturated solution of baryta added. The precipitate consisting of the silver salt of histidine is filtered off. Instead of adding an excess of baryta, Kossel and Pringle [76] direct that a suspension of barium carbonate be added to the neutral solution, the solution warmed on the steam-bath and then brought to the boiling-point. After cooling, the histidine silver is filtered off and washed free from nitric acid with baryta in the usual manner. The histidine silver precipitate is suspended in hot water, a small quantity of sulfuric acid added, and the precipitate decomposed with hydrogen sulfide. The silver sulfide is filtered off and a Kjeldahl nitrogen determination of an aliquot of the filtrate gives the amount of histidine. The histidine is isolated from the remainder of the solution as the dichloride. The yield of histidine dichloride is 75 to 80 per cent of the histidine estimated by the Kjeldahl determination. The histidine may also be isolated from this solution as the picrolonate, as suggested by Weiss.

The filtrate containing the arginine is saturated with baryta and the silver salt of arginine thus obtained is filtered off. After freeing the precipitate from nitric acid by careful washing with baryta water, it is suspended in water containing a small amount of sulfuric acid and decomposed with hydrogen sulfide as in the case of histidine. The silver sulfide is filtered and a total nitrogen determination of an aliquot of the filtrate gives the amount of arginine. The arginine is isolated from the remainder of the solution either as the nitrate, copper nitrate double salt, or as the picrolonate.

The filtrate, containing the lysine, from the precipitate of the silver salts of arginine and histidine is acidified with sulfuric acid and freed from silver by hydrogen sulfide. The lysine is precipitated, in the pres-

ence of 5 per cent sulfuric acid, as the phosphotungstate. An aqueous suspension of the lysine phosphotungstate is poured into boiling water and a hot saturated solution of baryta added in excess. The barium phosphotungstate is filtered off, the alkaline filtrate freed from baryta by means of carbon dioxide. The lysine is isolated from the solution and estimated as the picrate.

The amount of each of the three amino acids is determined, in practically all modifications of this method, from the total-nitrogen content of the solution which is assumed to contain nitrogen only in the form of the particular amino acid. Some loss is inevitable in the isolation of the pure compound and subsequent weighing. Osborne, Van Slyke, Brautlecht, and Vinograd,[77] however, have shown that, using extreme care, there is little loss, especially in the isolation of lysine picrate.

In the original procedure, after the histidine had been separated by precipitation from the arginine with silver nitrate, the histidine precipitate was decomposed and the histidine purified by again precipitatng with silver nitrate. Kossel and Patten [74] suggested the use of mercuric sulfate in the place of silver nitrate for this second precipitation. Osborne, Leavenworth and Brautlecht,[2] however, found it better to reverse the procedure and make the first precipitation with mercuric sulfate and the final one with silver nitrate. Any histidine not removed by mercuric sulfate was recovered by precipitation with silver nitrate.

Osborne and Harris [68] pointed out that the nitrogen precipitated by phosphotungstic acid under the conditions of the Hausmann method for the estimation of basic nitrogen agreed closely with the sum of that contained in the basic amino acids, arginine, histidine, and lysine. Osborne, Van Slyke, Leavenworth, and Vinograd [77] make use of this finding in their simplification of the Kossel method for the determination of these amino acids. In their modification the basic amino acids are first precipitated with phosphotungstic acid under the conditions of the Hausmann method as used by them with the modification that losses which might occur on decomposing the phosphotungstate precipitate were avoided by using the amyl alcohol-ether method of Van Slyke [80] for this decomposition. The arginine and histidine are later precipitated by baryta and silver nitrate, as in the Kossel method, and the lysine reprecipitated with phosphotungstic acid. The lysine phosphotungstate is also decomposed by the amyl alcohol-ether method and the lysine finally estimated by weighing as the picrate.

Osborne, Van Slyke, Leavenworth, and Vinograd [77] compared the results obtained by the Kossel method with those obtained by the Van

[80] Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxii, 281.

Slyke method.[81, 9, 80] Comparing the percentages of these bases, as determined directly by the Kossel method, with those calculated from the results of the Van Slyke method, it was found that the Van Slyke method always gave the higher results. In the case of arginine the agreement was fair but in the case of histidine and especially of lysine the differences were too great. Some differences are to be expected due to the fact that one is a direct and the other is an indirect method, but even when the utmost care was taken to prevent loss of lysine the per cent of this amino acid was considerably higher by the Van Slyke distribution method.

As before stated, the Kossel and Kutscher method and its modifications depend primarily upon the separation of the arginine and histidine by the difference in the solubility of their silver salts in neutral and alkaline solutions. Kossel and Edlbacher [82] in 1920 stated that a $N/50$ histidine solution reacts alkaline to phenolphthalein but not to thymolphthalein, while a $N/50$ solution of arginine reacts alkaline to both indicators. They state further that histidine is completely precipitated when the solution, containing an excess of silver nitrate, is made alkaline to phenolphthalein, but still acid to thymolphthalein, with barium hydroxide; *i.e.,* when the reaction lies between pH 8.3 and 9.3. Under these conditions it is claimed that all the histidine is precipitated by silver nitrate, while the precipitation of the silver salt of arginine has not commenced; when the reaction is made alkaline to thymolphthalein arginine silver is completely precipitated. Vickery and Leavenworth,[83] however, found that when histidine silver was precipitated in a solution made faintly alkaline to phenolphthalein, considerable amounts of arginine silver were invariably obtained at the same time. Nevertheless these authors feel that the quantitative separation of histidine and arginine, as their silver salts, is a problem in which hydrogen ion concentration plays a decisive part and that a separation will ultimately be effected by a proper control of this condition.*

[81] Van Slyke, D. D., *Ber. chem. Ges.,* 1910, xliii, 3170.
[82] Kossel, A., and Edlbacher, S., *Z. physiol. Chem.,* 1920, cx, 241.
[83] Vickery, H. B., and Leavenworth, C. S., *J. Biol. Chem.,* 1926, lxviii, 225.

* Vickery and Leavenworth (*J. Biol. Chem.,* 1928, lxxvi, 707) have recently made some noteworthy changes in this method. Histidine and arginine are separated on the basis that arginine silver is soluble and histidine silver is insoluble at a reaction of pH 7.0. The quantities of both bases are calculated from the respective weights of their dinitronaphtholsulfonates. Silver oxide is used in place of the silver nitrate, thus obviating the usual troublesome washing free from nitrate. Preliminary precipitaton with phosphotungstic acid is not recommended.

In a still more recent publication, Vickery and Leavenworth (*J. Biol. Chem.,* 1928, lxxix, 377) report the basic amino acid content of horse hemoglobin. They used a slight modification of the procedure just described, necessitated mainly by the high proportion of histidine to arginine found in hemoglobin. The silver salt of histidine was found to be most completely separated from that of arginine at a pH of 7.4. As in the above-described procedure, evidence as to actual composition of the histidine and arginine fractions was secured from the weights of their respective dinitronaphtholsulfonates and that of the lysine fraction from the weight of its picrate. By the aid of reprecipitations of precipitates and careful examinations of mother liquors, the accuracy of the results have been greatly enhanced. This

Fischer's Ester Method

Practically nothing was known concerning the quantitative occurrence of the monoamino acids in proteins until Emil Fischer [1] in 1901 introduced the method of fractional distillation of the ethyl esters under very low pressure. The method as proposed by Fischer was not considered by him to be a quantitative method for the determination of the amino acids, but rather a means for qualitative isolation of the amino acids for their identification or for the production of pure compounds. Slight modifications which have been made in the method by numerous investigators, particularly Abderhalden,[84] have made it more satisfactory for quantitative work, but numerous sources of error still prevent its use as an accurate determination of the amino acid content of proteins. The details of this rather tedious method have been described particularly well by Abderhalden,[84] by Osborne and Jones,[85] and by Plimmer.[73, pp. 33–50] The essential steps in the procedure are as follows:

I. *Hydrolysis.* In this method a large amount of protein material is necessary: at least three hundred grams are usually taken. The protein is hydrolyzed completely by boiling with sulfuric or hydrochloric acid in the usual manner. The humin substances are then removed by filtration.

II. *Separation of Glutamic Acid Hydrochloride.* In most cases it is convenient to separate much of the glutamic acid from the above filtrate at this point by concentrating under diminished pressure, saturating with dry hydrochloric acid gas, and then allowing to stand for several days at 0° when the glutamic acid hydrochloride crystallizes out. The method of isolating glutamic acid as the hydrochloride was first used by Hlasiwetz and Habermann.[8]

III. *Esterification.* The combined mother liquors from the glutamic acid hydrochloride are evaporated under diminished pressure to a syrup, taken up in absolute alcohol, again evaporated to a syrup, and the same procedure repeated until the water is removed as completely as possible. The amino acids in the syrup are then esterified by taking up in absolute alcohol and saturating the solution with dry hydrochloric acid gas. The water formed during the esterification process prevents the reaction from going to completion. It is therefore necessary to remove the water by concentration *in vacuo* and re-esterify as before. It is sometimes advisable to repeat this procedure a third time.

method of Vickery and Leavenworth is without doubt one of the most accurate procedures for the determination of the bases, arginine, histidine and lysine, yet described.

[84] Abderhalden, E., "Handbuch der Biochemischen Arbeitsmethoden," 1910, Band II, 470.

[85] Osborne, T. B., and Jones, D. B., *Am. J. Physiol.*, 1910, xxvi, 212.

According to Osborne and Jones,[85] the esterification may be effected more quickly and more completely by the method proposed by Phelps and Tillotson[86] for esterifying organic acids. In this procedure the concentrated solution of the amino acid hydrochlorides is dissolved in alcoholic hydrochloric acid and zinc chloride is added as a catalyst. Vapors of absolute alcohol are passed continuously through the solution kept slightly above 100°. At this temperature the water is removed as fast as it is formed and complete esterification is obtained in one operation.

Probably the most quantitative method yet proposed for amino acid esterification is that of Foreman,[87] who converts the amino acids into their lead salts which are dried, suspended in alcohol and esterified by saturating with hydrochloric acid gas. This is especially effective if the dibasic amino acids have been previously removed.

IV. *Isolation of Glycine Ethyl Ester Hydrochloride.* If it is known that much glycine is present, it may be taken out at this point by partial concentration *in vacuo* at 40° or less, seeding with a crystal of glycine ester hydrochloride, and allowing to stand at 0° for several hours. Although the glycine ethyl ester hydrochloride is difficultly soluble in alcohol, a second crop of crystals, and perhaps a third, may be obtained on further concentration of the mother liquors and saturation with hydrochloric acid gas. After this procedure the remaining amino acids are usually re-esterified.

V. *Liberation of the Free Amino Acid Esters.* The esters are liberated from their hydrochlorides in one of the following ways:

(*a*) Sodium hydroxide. The amino acid esters may be freed from their hydrochlorides by the addition of strong sodium hydroxide solution while the solution is thoroughly cooled in a freezing mixture, salted out with solid potassium carbonate, and extracted with ether. A large amount of ether is necessary for this extraction and careful manipulation is required in order to obtain the best yield of free esters. Throughout the procedure thorough cooling is essential. An objection to this method is that some of the esters are saponified and it is often necessary to repeat the processes of esterification and subsequent liberation of the esters two or three times. By this method of freeing the esters from their hydrochlorides, neither the tyrosine, which remains behind combined with the alkali, the hydroxyproline, nor the diamino acids, which are difficultly soluble in ether, are obtained in the ether extract. A final extraction with chloroform takes out the tyrosine and

[86] Phelps and Tillotson, *A. J. Sci.*, 1907, xxiv, 194. Quoted from Osborne and Jones.[85]
[87] Foreman, F. W., *J. Agr. Sci.*, 1913, iv, 431; *Biochem. J.*, 1919, xiii, 378.

at the same time removes, more completely than did the ether, some of the other esters.

(*b*) Sodium ethylate. A second method of liberating the esters from their hydrochlorides is by means of sodium ethylate. In this method there is no loss by saponification but, according to Plimmer, "the yield of higher boiling fractions is not so great on account of the more complex nature of the mixture of esters."

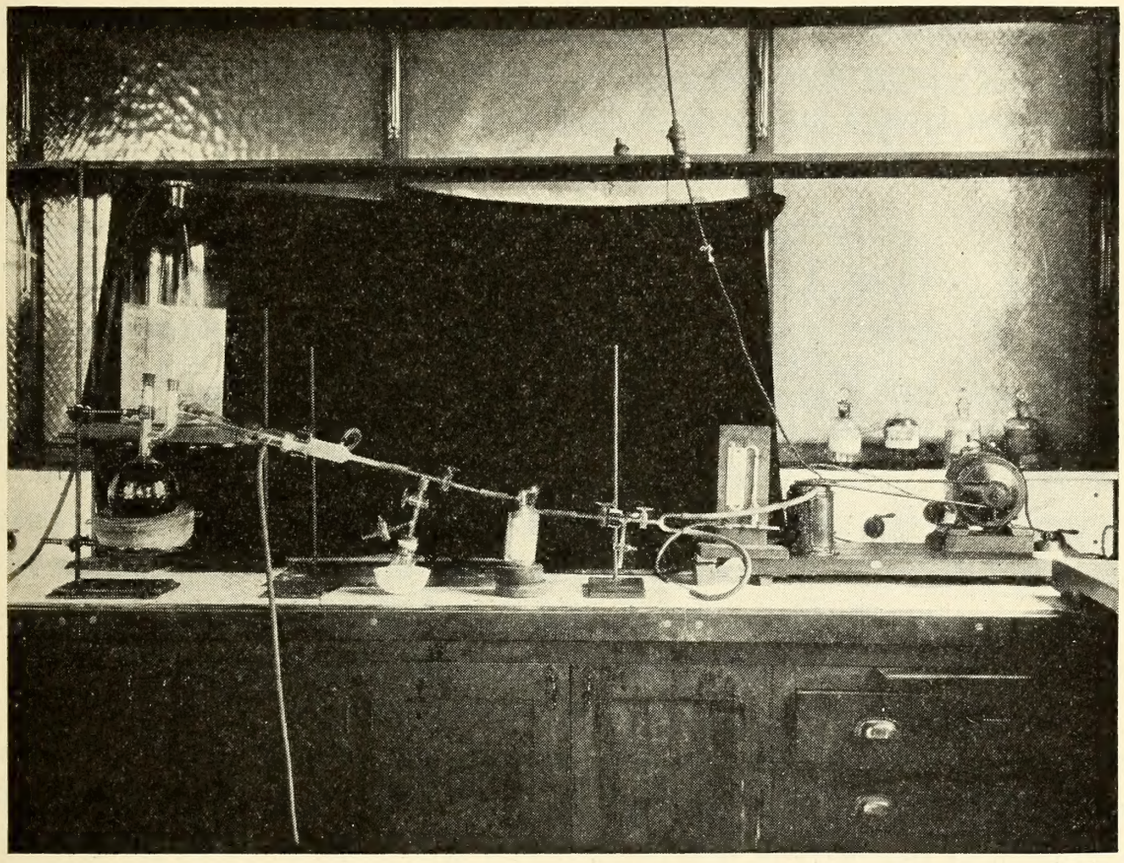


FIG. 18.—The Fischer-ester distillation apparatus for the determination of the amino-acid contents of proteins, as used in the Bureau of Chemistry and Soils of the United States Department of Agriculture. (Secured through the courtesy of Dr. D. B. Jones.)

(*c*) Barium hydrate. A third method used for freeing the esters is the barium hydrate method.[88] This method was proposed by Levene and used to good advantage by Levene and Van Slyke. Barium hydrate has the advantage over a stronger alkali in that no saponification takes place, less heat is evolved during neutralization, and it is more readily removed when a second esterification is necessary.

(*d*) Ammonia. Ammonia has also been suggested [89] as a reagent

[88] Levene, P. A., *J. Biol. Chem.*, 1905. i, 45; Levene, P. A., and Alsberg, C. L., *ibid.*, 1906, ii, 127; Levene, P. A., and Van Slyke, D. D., *Biochem. Z.*, 1908, xiii, 442; *J. Biol. Chem.*, 1909, vi, 419.

[89] Pribram, B. O., *Monatsh. Chem.*, 1910, xxxi, 51.

for the liberation of the amino acid esters from their hydrochlorides. Abderhalden has found this method very serviceable.

(*e*) Lead oxide. Another method involves the use of lead oxide.[90]

VI. *Fractional Distillation of the Esters under Low Pressures.* The brown oil, which remains after the distillation of the solvent used in extracting the esters liberated by either of the above methods, is distilled fractionally. It is fractionated first at a pressure of 10 to 12 mm., and then at 0.5 mm. Different investigators collect different fractions and these depend, to some extent, on the amounts of the particular amino acids present in the protein under investigation. The following table, taken from Plimmer, [73, p. 42] shows the fractions usually collected and the esters that may be found in each fraction:

	Temperature	Pressure	Esters of
Fraction I.	To 60° (vapor)	10 mm.	Glycine, alanine, leucine, proline.
Fraction II.	60-90° (vapor), 100° (water-bath)	10 mm.	Valine, leucine, proline.
Fraction III.	100° (water-bath)	0.5 mm.	Leucine, proline.
Fraction IV.	100-160° (oil-bath)	0.5 mm.	Phenylalanine, glutamic acid, aspartic acid, serine.

VII. *The Isolation of the Individual Amino Acids.* The separation and estimation of the individual amino acids in each fraction is a tedious process. The procedure is different for each amino acid, involving the characteristic physical and chemical properties of each.

The technical difficulties of the ester method are obviously very great and the values obtained are approximate and represent the minimum values only. A critical discussion of these difficulties and a consideration of the various sources of loss is given by Osborne and Jones.[28] Even from a known mixture of amino acids only from 60 to 70 per cent can be recovered. Although the method cannot be considered as quantitative, it gives a good general idea of the amounts of each of the various amino acids present in a protein and by its use clear-cut and important differences among proteins have been obtained.

Siegfried's Carbamino Method

The carbamino reaction discovered by Siegfried in 1904, previously referred to [31, 32] (page 101), may be used in getting an idea of the composition of complex amino acid mixtures. The calcium salts of the carbamino acids are soluble in water, but on boiling are broken down, precipitating the calcium carbonate and freeing the amino acids. By determining the carbon dioxide, from the amount of calcium carbonate

[90] Zelinsky, N., Annenkoff, A., and Kulikoff, J., *Z. physiol. Chem.*, 1911, vii, 459.

precipitated, and the nitrogen in the filtrate or in the original solution of carbamino salts, a ratio between the amounts of carbon dioxide and nitrogen in the carbamino acid can be obtained. This ratio is significant. The CO_2/N quotient for the monoamino acids is 1, for histidine 3, for arginine 4. The determination of this quotient in a protein hydrolysate, therefore, may indicate important differences in proteins.

The barium salts of many of the carbamino acids are insoluble in water and thus the reaction may be used for the separation of one amino acid from another.[91] Neuberg and Kerb [92] used the mercury salts of the carbamino acids as a means of separating the amino acids. This apparently simple method has been little used by investigators other than Siegfried and his co-workers.

Sörensen's Titration Method with Formalin

The amino acids in solution are so nearly neutral in reaction that they cannot be titrated with alkali like ordinary acids. Sörensen,[29] however, in 1907, taking advantage of a reaction discovered by Schiff,[93] showed that, by first coupling the amino groups with formaldehyde, the free carboxyl groups could then be accurately titrated with standard alkali using phenolphthalein as indicator. The reaction between the amino acid and formaldehyde results in the formation of a methylene derivative, which now acts as a free carboxylic acid:

$$\begin{array}{l} \mathrm{R.CH.COOH} \\ \quad\mid \\ \quad\mathrm{NH_2} \end{array} + \mathrm{CH_2O} \longrightarrow \begin{array}{l} \mathrm{R.CH.COOH} \\ \quad\mid \\ \quad\mathrm{N{:}CH_2} \end{array} + \mathrm{H_2O}$$

$$\begin{array}{l} \mathrm{R.CH.COOH} \\ \quad\mid \\ \quad\mathrm{N{:}CH_2} \end{array} + \mathrm{KOH} \longrightarrow \begin{array}{l} \mathrm{R.CH.COOK} \\ \quad\mid \\ \quad\mathrm{N{:}CH_2} \end{array} + \mathrm{H_2O}$$

The general procedure involves, first, the removal of coloring matter, ammonia, phosphates, and other substances which might interfere with the reactions; second, the neutralization of the amino groups with neutralized formalin; and third, the titration with standard baryta or sodium hydroxide, using phenolphthalein as indicator. The acidity as shown by the titration is a measure of the amount of amino acid nitrogen present. If the amino acid be known its amount in the solution can be calculated. If the amino acid be unknown, or if a mixture of amino acids be present, the amount is best expressed in terms of 0.1 *N* acid, or in terms of α-amino nitrogen.

Ammonia likewise reacts with formaldehyde with the formation of hexamethylene-tetramine, hence the formaldehyde titration in the pres-

[91] Siegfried, M., and Schmitz, M., *Z. physiol. Chem.*, 1910, lxv, 295; Abderhalden, E., and Kautsch, K., *ibid.*, 1910, lxviii, 487; Siegfried, M., and Schutt, E., *ibid.*, 1912, lxxxi, 260.
[92] Neuberg, C., and Kerb, J., *Biochem. Z.*, 1912, xl, 498.
[93] Schiff, H., *Ann. Chem.*, 1900, cccx, 25, 301; 1902, cccxxv, 348.

ence of ammonia gives results which include both amino acid and ammonia nitrogen. Ammonia may be determined and a correction applied, or the ammonia may be removed by mercuric chloride,[94] by phosphotungstic acid,[95] or by phosphotungstic acid and magnesium chloride.[96] Phosphates also interfere by obscuring the end-point. They may be removed by the addition of barium salts.

Sörensen and Jessen-Hansen [30] have advocated the precipitation of silver chloride in highly colored hydrolysates in order to remove the coloring matter. Malfatti [94] objected to the use of litmus as indicator in one part of the procedure (before the addition of formalin) and phenolphthalein in another part (after the addition of formalin). Henriques and Sörensen [97] show, however, that these titrations are two distinct steps, the former being for the sake of neutralization and the latter being the true formaldehyde titration. Since the reactions are reversible and the desire is to carry the reactions to the right as far as possible, titration to the second or third state with phenolphthalein is recommended. Litmus changes at about 10^{-7}, phenolphthalein becomes pale rose at 10^{-8} to $10^{-8.4}$, a distinct red at $10^{-8.7}$ to $10^{-8.8}$, and a strong red color at $10^{-9.0}$ to $10^{-9.1}$. For the preliminary neutralization the object is quite different; namely, to make sure that there shall be equivalent amounts of free basic and acidic groups present.

The method is, with some modifications involving the preparation of the solution to be titrated, applicable to the determination of amino acids in any medium. Malfatti,[94] Henriques,[98] Yoshida,[99] de Jager,[100] and Henriques and Sörensen [97, 101] applied the method to urine analysis. Costantino,[102] Labbé and Debré,[103] and Lematte [104] used the method for the determination of the amino acids in blood; Delaunay,[105] von Fürth and Schwarz,[106] Buglia and Costantino,[107] and Okuda [108] in animal tissues; and Bailly [109] and Adler [110] in plant tissues.

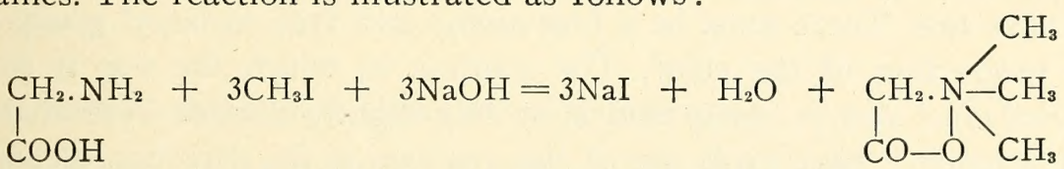
[94] Malfatti, H., *Z. physiol. Chem.*, 1909, lxi, 499.
[95] Benedict, S. R., and Murlin, J. R., *Proc. Soc. Exp. Biol. and Med.*, 1912, xviii, 109; *J. Biol. Chem.*, 1913, xvi, 385.
[96] Lematte, L., *Bull. sci. pharmacol.*, 1913, xx, 577, 647; through *J. Chem. Soc., Abs.*, 1914, cvi, ii, 305; *Chem. Abs.*, 1914, viii, 1801.
[97] Henriques, V., and Sörensen, S. P. L., *Z. physiol. Chem.*, 1909, lxiii, 27.
[98] Henriques, V., *Z. physiol. Chem.*, 1909, lx, 1.
[99] Yoshida, T., *Biochem. Z.*, 1910, xxiii, 239.
[100] de Jager, L., *Z. physiol. Chem.*, 1909, lxii, 333; 1910, lxvii, 105.
[101] Henriques, V., and Sörensen, S. P. L., *Z. physiol. Chem.*, 1910, lxiv, 120.
[102] Costantino, A., *Biochem. Z.*, 1913, li, 91; 1913, lv, 402, 419.
[103] Labbé, H., and Debré, R., *Compt. rend. Soc. Biol.*, 1913, lxxiv, 199, 563.
[104] Lematte, L., *Compt. rend. Acad. Sci.*, 1914, clviii, 1379.
[105] Delaunay, H., *Compt. rend. Soc. Biol.*, 1910, lxix, 592, 594.
[106] von Fürth, O., and Schwarz, C., *Biochem. Z.*, 1911, xxx, 413.
[107] Buglia, G., and Costantino, A., *Z. physiol. Chem.*, 1912, lxxxi, 109, 130, 145, 154; 1912, lxxxii, 439; 1913, lxxxiv, 243.
[108] Okuda, Y., *Orig. Com. 8th Internat. Congress Appl. Chem.*, 1912, xviii, 275.
[109] Bailly, O., *Bull. sci. pharmacol.*, 1912, xviii, 702; through *Chem. Zentbl.*, 1912, lxxxiii, part 2, 1640; and other abstract journals.
[110] Adler, L., *Z. ges. Brauw.*, 1914, xxxvii, 105, 117, 129; through *Chem. Abst.*, 1914, viii, 2025.

Kossel and Gawrilow [111] report that proteins, in which the lysine group is present, titrate like amino acids, and that others do not. Several protamines of the salmine group do not, nor do zein and hordein. Obermayer and Wilheim [112] also find proteins titratable when no free amino acid is present. They propose a theory as to the complexity of proteins based on the idea that this titration determines the amount of amino acid in the exposed positions of proteins and enables the computation of the ratio of total nitrogen to amino nitrogen (which they call the amino index).

Clementi [113] raises the question whether substitution in the amino groups prevents the determination of the same by this method. Sarcosine, which is methyl glycine, reacts like glycine only that the values are somewhat lower, and the titration should be carried to intense red.

Engeland's Exhaustive Methylation Method

In contradistinction to the method of Sörensen, in which the basicity of the amino groups is neutralized by coupling with formaldehyde,* Engeland's method [114, 115, 116] involves the strengthening of the basicity, since in general, organic bases are easier to obtain analytically pure than are organic acids. Engeland makes use of the Hofmann methylation reaction which Gries [117] first applied to the amino acids, forming betaine from glycine. The amino acids are methylated in alkaline solution with methyl iodide or dimethyl sulfate and converted into the corresponding betaines. The reaction is illustrated as follows:



These compounds are all distinguished by the crystallizing capacity of their double salts with the heavy metals, such as the aurates. The method of exhaustive methylation can be applied with advantage to a mixture of amino acids. The corresponding betaines can be separated by the different solubilities of their double salts with mercury, platinum, and gold chlorides. These double salts may be isolated, weighed, and identified, and the amount of amino acid originally present computed from the amount of betaine.

[111] Kossel, A., and Gawrilow, N. *Z. physiol. Chem.*, 1912, lxxxi, 274.
[112] Obermayer, F., and Wilheim, R., *Biochem. Z.*, 1912 xxxviii, 331: 1913, 1, 369.
[113] Clementi, A., *Atti R. Acad. Lincei, Rend. Cl. Sci. Fis., Mat. e Nat.*, 5. series, 1915, xxiv, I. 352; through *Exp. Sta. Rec.*, 1916, xxxv, 315.
* According to Harris this explanation is not entirely correct; see page 134.
[114] Engeland, R., *Ber. chem. Ges.*, 1909, xlii, 2962.
[115] Engeland, R., and Kutscher, F., *Z. Biol.*, 1912-13, lix, 415.
[116] Engeland, R., *Z. Biol.*, 1914, lxiii, 470.
[117] Gries, P., *Ber. chem. Ges.*, 1875, viii, 1406.

Engeland isolated from casein [114] *l*-methyl-hygric acid, which is derived from proline, trimethyl leucine, trimethyl valine, betaine, and trimethyl alanine. Subsequently he [118] prepared trimethyl phenylalanine and dimethyl glutamic acid. In 1914 Engeland [116] isolated methyl-hygric acid from the products of hydrolysis of spongin and in 1922 [119] he applied the method to the determination of proline in glutin and elastin.

The products resulting from the methylation of proteins and protein hydrolytic products have been studied by Skraup and his co-workers,[120] Herzig and his co-workers,[121, 122] Edlbacher,[123] and others. Herzig and Landsteiner [122] recommended the use of diazomethane as the agent for methylating the amino acids.

The procedure is laborious and apparently offers no advantages over the ester method, but may be of value in the isolation of certain products resulting from the ester method for which there are no other satisfactory methods available.

The Ninhydrin Reaction

Ruhemann [33] discovered that all acids containing a free amino group in the alpha position reacted with triketo-hydrindene hydrate, $C_6H_4\langle{}^{CO}_{CO}\rangle C.(OH)_2$, (commercially known as ninhydrin) with the production of an intense blue color. β- and γ-amino acids hardly react at all, and α-amino acids, when substituted on the amino group, give a negative test. There must be a free amino and free carboxyl group for the production of the color. The solution to which the test is to be applied must also be nearly neutral or only slightly alkaline. Abderhalden and Schmidt [34] have made use of this reaction in the detection of amino acids and it is one of the most sensitive reactions for this purpose. By means of this reagent it is possible to show the presence of amino acids in fresh urine and in protein-free blood serum. As far as is known, there is no amino acid obtained from protein hydrolysis which does not give the test.

Herzfeld [124] developed a spectrophotometric method for the quantitative application of the method. Harding and MacLean [35, 36] adopted the method for use with a Duboscq colorimeter instead of a spectro-

[118] Engeland, R., *Ber. chem. Ges.*, 1910, xliii, 2662.
[119] Engeland, R., *Z. physiol. Chem.*, 1922, cxx, 130.
[120] Skraup, Zd. H., and Krause, E., *Monatsh.*, 1909, xxx; 447; Skraup, Zd. H., and Böttcher, B., *ibid.*, 1910, xxxi, 1035.
[121] Herzig, J., *Z. physiol. Chem.*, 1920, cxi, 223.
[122] Herzig, J., and Landsteiner, K., *Biochem. Z.*, 1920, cv, 111.
[123] Edlbacher, S., *Z. physiol. Chem.*, 1919, cvii, 52; 1919, cviii, 287; 1920, cx, 153; 1920, cxii, 80.
[124] Herzfeld, E., *Biochem. Z.*, 1914, lix, 249.

photometer and showed that the method is of value in following the course of protein hydrolysis.

Neuberg [125] criticized the specificity of the ninhydrin reaction for amino acids and showed that ammonium salts, in general, gave the test. Harding and Warneford [126] and Harding and MacLean [127] have examined the ninhydrin reaction with amino acids, ammonium salts, amines, and amides. They found that the ninhydrin reaction was very sensitive in the case of all the amino acids and that ammonium salts also gave a positive reaction, provided the concentrations were sufficiently high. The reaction with amines was found to depend upon the concentration and upon their constitution, all bases of the type $R.CH_2NH_2$, and all bases of the type $R.CH(NH_2)R$ where one radical was negative, gave positive results. Amides gave negative results. Harding and MacLean [127] point out that the use of ninhydrin both as a qualitative and quantitative reagent is, therefore, open to serious objections. It is only in the absence of large amounts of ammonium salts and bases that the method is reliable. These conditions are fulfilled in the hydrolysis of protein by pancreatic enzymes. Harding and Warneford [126] suggested that the mechanism of the reaction between ninhydrin and the amino acids could be explained by adopting the hypothesis of Dakin and Dudley [128] which supposes the decomposition of the amino acid into ammonia and a glyoxal, the latter acting as a reducing agent.

The applicability of the ninhydrin reaction has also been studied by Abderhalden and Lampe,[129] Koritschoner and Morgenstern,[130] Riffart,[131] and others.

Van Slyke's Nitrogen Distribution Method

Principle. In an attempt to overcome the necessity of using such large amounts of protein material as are required by the Kossel and Kutscher and by the Fischer methods (20 to 30 grams being required by the former and from 300 to 400 grams by the latter), and at the same time to obtain results more nearly quantitative than is possible by these methods, Van Slyke [81, 9, 80] in 1910 and 1911 proposed his now well-known nitrogen distribution method. This method represents a departure from the Kossel and Kutscher and the Fischer methods in that, instead of determining the various amino acids directly, their quantities are calculated from a determination of the chemical groups characteristic

[125] Neuberg, C., *Biochem. Z.*, 1913, lvi, 500; 1914, lxvii, 56.
[126] Harding, V. J., and Warneford F. H. S., *J. Biol. Chem.*, 1916, xxv, 319.
[127] Harding, V. J., and MacLean, R. M., *J. Biol. Chem.*, 1916, xxv, 337.
[128] Dakin, H. D., and Dudley, H. W., *J. Biol. Chem.*, 1913, xv, 127.
[129] Abderhalden, E., and Lampe, A. E., *Z. physiol. Chem.*, 1912, lxxxi, 473; 1913, lxxxv, 136.
[130] Koritschoner, R., and Morgenstern, O., *Biochem. Z.*, 1919, xciii, 172.
[131] Riffart, H., *Biochem. Z.*, 1922, cxxxi, 78.

of these acids. Since, by this method, similarly constituted amino acids are grouped together, comparatively few of the individual acids may be determined. This number, however, may be increased by supplementing the Van Slyke method with methods suitable to the estimation of certain of the individual amino acids.

Van Slyke's method is based on the reaction between aliphatic amino groups and nitrous acid whereby the nitrogen of the amino group is liberated as gaseous nitrogen. The reaction is illustrated by the following examples:

$$R.NH_2 + HNO_2 = R.OH + H_2O + N_2$$

$$\begin{array}{l} CH_2.COOH \\ | \\ NH_2 \end{array} + HNO_2 = \begin{array}{l} CH_2.COOH \\ | \\ OH \end{array} + H_2O + N_2$$

Of the total nitrogen evolved, half is derived from the amino group and half from the nitrous acid. According to Van Slyke:[132] "Every known amino-acid obtained from protein by acid hydrolysis reacts quantitatively with one and only one nitrogen atom, except lysin, which reacts with two, and proline and oxyproline, which do not react at all. All the amino acids react with all their nitrogen, except tryptophan, which reacts with one-half, histidin with one-third, arginine with one-fourth, and proline and oxyproline with none."

Van Slyke's method makes use of two procedures: (*a*) The separation of the monoamino from the diamino acids by precipitation of the latter with phosphotungstic acid, as in the modified[68] Hausmann method;[40] and (*b*) the quantitative determination of the aliphatic amino nitrogen by the nitrous acid method developed by Van Slyke.[132, 81, 133, 134, 135] In the application of the Van Slyke method to the analysis of proteins, the nitrogen distributed among the following groups is determined:

I. Amide nitrogen (ammonia)

II. Precipitated by phosphotungstic acid:
  (a) 100 per cent of N as NH_2
    (i) Cystine (contains sulfur)
    (ii) Lysine (contains no sulfur)
    Sulfur content gives cystine; lysine by difference.
  (b) Non-amino N
    (iii) Arginine (contains ¾ of its N in non-amino form)
    (iv) Histidine (contains ⅔ of its N in non-amino form)
    Arginine evolves half of its N as NH_3 when boiled with alkali; histidine by difference.

[132] Van Slyke, D. D., *J. Biol. Chem.*, 1911, ix, 185.
[133] Van Slyke, D. D., *Proc. Soc. Exp. Biol. Med.*, 1909, vii, 46; *J. Biol. Chem.*, 1910, vii, Proc., p. xxxiv; *Proc. Am. Soc. Biol. Chem.*, 1910, i, 247.
[134] Van Slyke, D. D., *Ber. chem. Ges.*, 1911, xliv, 1684.
[135] Van Slyke, D. D., *J. Biol. Chem.*, 1912, xii. 275.

III. Not precipitated by phosphotungstic acid:
  (a) 100 per cent N as NH_2
    (i) Glycine, phenylalanine, aspartic acid, etc.
  (b) Non-amino N
    (ii) Proline and hydroxyproline
    (iii) Tryptophane (contains ½ of its N in non-amino form)

Thus, of the possible nineteen data (nineteen amino acids are definitely known), the following seven may be obtained by the Van Slyke analysis: 1, Ammonia nitrogen; 2, arginine nitrogen; 3, cystine nitrogen; 4, histidine nitrogen; 5, lysine nitrogen; 6, amino nitrogen in the filtrate from the phosphotungstic acid precipitate; 7, non-amino nitrogen in the filtrate from the phosphotungstic acid precipitate.

Apparatus. Sachsse and Kormann[136] in 1875 made use of the reaction between aliphatic amino groups and nitrous acid for the quantitative determination of amino acids, applying potassium nitrite and sulfuric acid and collecting the gases over ferrous sulfate and potassium hydrate, which were supposed to absorb all gases except nitrogen. Brown and Millar[137] examined this method thoroughly and pointed out several sources of error, the most likely of which were imperfect washing of air from the apparatus and not a quantitative absorption of nitric oxide by ferrous sulfate. They removed the air from the apparatus with a current of carbon dioxide and subsequently absorbed this gas in potassium hydroxide solution. The excess of nitric oxide was removed by means of oxygen and then the excess of oxygen with pyrogallol.

The original apparatus used by Van Slyke was very similar to that used by Sachsse and Kormann and by Brown and Millar. Klein[138] improved the apparatus and Van Slyke[135] soon adopted this modification. Van Slyke[139] described a smaller form of the apparatus for use in the analysis of amino acids in blood, tissues, etc., and later[140] described a further improved form of micro apparatus. The nitrous acid is obtained by the interaction of sodium nitrite and acetic acid. The air in the apparatus is displaced by the nitric oxide produced by the spontaneous decomposition of nitrous acid. The nitric oxide and any possible carbon dioxide are absorbed in alkaline permanganate and the present form of the apparatus is nicely adjusted for the easy introduction of the solutions as called for and for the accurate measurement of the nitrogen evolved. The volume of nitrogen measured is corrected for temperature and pressure in the usual manner; one-half of this volume representing the nitrogen from the amino groups. The weight

[136] Sachsse, R., and Kormann, W., *Landw. Versuchsstat.*, 1875, xvii, 321; *Z. anal. Chem.*, 1875, xiv, 380.
[137] Brown, H. T., and Millar, J. H., *Trans. Guinness Laboratory*, 1903, i, 29.
[138] Klein, D., *J. Biol. Chem.*, 1911, x, 287.
[139] Van Slyke, D. D., *J. Biol. Chem.*, 1913, xvi, 121.
[140] Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxiii, 407.

of the volume of nitrogen may be calculated or may be readily taken from a table prepared by Van Slyke [135] and reproduced in most physiological textbooks. The whole process takes but a short time.

Procedure. The details of the procedure and the management of the apparatus are completely described by Van Slyke [9] and reproduced in most physiological texts so that only a general outline of the procedure will be given here.

Usually 3- or 6-gram portions of protein in duplicate are completely hydrolyzed by boiling with 20 per cent hydrochloric acid. Ammonia is then determined in the hydrolysate by distillation with lime under diminished pressure. During the distillation the humin or melanin, which was formed during hydrolysis, is absorbed by the undissolved lime. The solution is filtered and total nitrogen determined in the residue; this gives the humin or melanin nitrogen. The bases are then precipitated with carefully purified phosphotungstic acid, the conditions of precipitation being much the same as those used by Osborne and Harris [68] in their modification of the Hausmann method. In the washing of the phosphotungstic acid precipitate it is necessary to follow the directions carefully because of the double danger of not washing out all the mother liquors and dissolving appreciable quantities of the bases. The original directions [9] for the filtration and washings have been modified successfully by Hartley [141] and by Van Slyke.[80]

The phosphotungstic acid precipitate of the bases is decomposed by dissolving in sodium hydroxide and precipitating the phosphotungstic acid with barium chloride, or better by the hydrochloric acid-amyl alcohol-ether method suggested by Van Slyke [80] in 1915. The respective amounts of cystine, arginine, histidine and lysine are determined in the solution of the bases by making use of the different chemical behaviors of these amino acids. For the estimation of arginine use is made of the action of strong alkali, whereby a molecule of arginine gives one molecule each of ornithine and urea, the latter being decomposed by the boiling alkali into ammonia, which is distilled and caught in standard acid during the operation. Plimmer [142] has shown that weaker alkali than that recommended by Van Slyke may be used to advantage. Cystine is estimated by a determination of the total sulfur content by Denis' modification [143] of Benedict's method.[144] By subtracting from the total nitrogen of the bases (determined by the Kjeldahl method), the total amino nitrogen of the bases (determined by the nitrous acid

[141] Hartley, P., *Biochem. J.*, 1914, viii, 541.
[142] Plimmer, R. H. A., *Biochem. J.*, 1916, x, 115.
[143] Denis, W., *J. Biol. Chem.*, 1910, viii, 401.
[144] Benedict, S. R., *J. Biol. Chem.*, 1909, vi, 363.

method), the total non-amino nitrogen is obtained. This is contained in the arginine and histidine. Since three-fourths of the arginine nitrogen and two-thirds of the histidine nitrogen are in this form, the total non-amino nitrogen (D) minus three-fourths of the arginine nitrogen represents two-thirds of the histidine nitrogen. Thus,

$$\text{Histidine N} = \tfrac{3}{2}\ (\text{D} - \tfrac{3}{4}\ \text{Arginine N})$$
$$= 1.5\ \text{D} - 1.125\ \text{Arginine N}\ *$$

The lysine is now calculated by difference:

$$\text{Lysine N} = \text{Total N} - (\text{Arginine N} + \text{Cystine N} + \text{Histidine N})$$

The total nitrogen and the amino nitrogen of the monoamine acids are obtained by the Kjeldahl and nitrous acid methods, respectively, on aliquots of the solution of the filtrate from the phosphotungstic acid precipitate.**

Corrections. In the determination of the hexone bases by the Van Slyke method certain corrections are to be made. Because of appreciable solubilities of the phosphotungstates of the bases, a correction for the amounts of nitrogen from each of the bases is made. When the precipitation has been carried out according to the directions, Van Slyke [9] has shown that these corrections may be considered as constants and may be taken from a table prepared by him. In the analysis of proteins containing large amounts of cystine, *i.e.*, in the case of keratins, two additional corrections are necessary. First, cystine evolves approximately 18 per cent of its nitrogen as ammonia during the arginine determination and thus, from the amount of cystine present, a correction factor must be calculated and applied to the ammonia found in the arginine determination. Second, Van Slyke [132] found that cystine behaves abnormally when treated with nitrous acid, in that the gas evolved is 107 per cent of the theoretical. Thus, in the estimation of the amino nitrogen of the bases the volume of gas must be correspondingly reduced. Glycine also behaves in a similar manner; it evolves 103 per cent of the theoretical amount of nitrogen.

Accuracy. Van Slyke [9] tested the method upon individual amino acids, upon a mixture of amino acids, and upon some typical proteins. In the tests upon individual amino acids, the agreement between the nitrogen figures found and those calculated from the amino acids present is fairly satisfactory, provided the solubility corrections for the bases

* In the original paper [9] Van Slyke gave the formula: 1.667 D—1.125 Arginine. This was later corrected by him.[145]

[145] Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxiii, 411.

** Plimmer and Rosedale (*Biochem. J.*, 1925, xix, 1015) have modified the Van Slyke procedure so as to determine arginine, histidine, and lysine without the removal of phosphotungstic acid and Plimmer and Lowndes (*Biochem. J.*, 1927, xxi, 247) have added the determination of cystine in this shortened procedure.

are applied. Cystine, however, even when tested alone, gave low results, which were due to partial destruction of this acid during hydrolysis. Van Slyke [9] showed that the destruction of this amino acid, when boiled with 20 per cent hydrochloric acid, depends upon the time of boiling; 41 per cent being destroyed in 16 hours and 50 per cent in 24 hours.

The artificial mixture of amino acids tested included aspartic acid, glutamic acid, proline, hydroxyproline, phenylalanine, tyrosine, alanine, glycine, valine, leucine, cystine, histidine, arginine, lysine and ammonium sulfate. While the results obtained by the analysis of this unboiled mixture of pure amino acids agreed closely with the actual amounts taken, the test, as Van Slyke suggested but never carried out, would have been more significant if tryptophane had been included and the mixture boiled for 20 to 24 hours with 20 per cent hydrochloric acid as is customary in protein analysis. This is especially significant, since it has been shown repeatedly that tryptophane is the source of considerable error in the method when applied to proteins containing this amino acid.

From analyses of typical proteins, Van Slyke [9] showed what differences were to be expected between duplicates. The maximum differences between duplicates varied from 0.11 (expressed in percentages of the total nitrogen) for cystine nitrogen to 2.14 for histidine nitrogen. The average differences between duplicates varied between 0.05 for cystine nitrogen and 0.79 for histidine nitrogen. The maximum difference of 2.14 for histidine was obtained on edestin and this percentage was more than twice as great as the next highest difference of 0.93 obtained for histidine. Van Slyke states that the latter figure probably represents as large a difference as would normally be expected. Since the amounts of some of the amino acids in some of the proteins examined were small, the significance of these differences is better appreciated when expressed as a percentage. The average percentage differences (difference between duplicates uncorrected for solubility expressed as a percentage of the average) in the six proteins analyzed by Van Slyke were 7.6 for cystine, 4.9 for arginine, 18 for histidine, and 18 for lysine. These percentages indicate that for histidine and lysine especially the method is far from quantitative.

Analyses of the same protein by different investigators, or even by the same investigator have given results that are not as concordant as is desired. Van Slyke in 1910 [81] reported an analysis of casein which was quite different from a subsequent analysis reported by him [146] in 1914. Although the first series of results were obtained with the older

[146] Van Slyke, D. D., *J. Biol. Chem.*, 1914, xvi, 531.

form of apparatus, the differences in results cannot be attributed solely to the slight differences in technic used. The following tabulation shows the results obtained. The results are expressed in percentages of the total nitrogen.

Analyses of Casein Reported by Van Slyke.[81, 146]

	1910	1914	Per Cent Difference from the Average
Cystine N	1.95	0.20	162
Arginine N	7.51	7.41	1.3
Histidine N	4.24	6.21	38
Lysine N	7.86	10.30	27

Since cystine is destroyed to an immeasurable degree during hydrolysis (less than one-half remaining in the phosphotungstic acid precipitate), the value of this determination as a measure of the cystine content of the original protein is slight. Arginine, apparently, can be determined with a good degree of accuracy, while histidine and lysine can be determined with only a moderate degree of accuracy.

Plimmer [72, p. 107] states that the accuracy of the method is borne out by the fact that by summation of all the nitrogen fractions the total nitrogen is recovered to within 1 per cent. On the contrary, this fact simply indicates that the methods used for the determination of the total and the amino nitrogen are accurate; the manner in which the method distributes the nitrogen among the different fractions is obviously without any influence on the total recovered.

That the phosphotungstates of the bases are appreciably soluble is recognized by Van Slyke and it is customary to apply a correction to each determination of total and amino nitrogen. These corrections apply to all solutions, regardless of the percentages of bases present, as long as they are precipitated in 200 cc. of solution according to the conditions of the Van Slyke procedure. Concerning the application of these corrections, Van Slyke [9] says, "It is true that the concentration of phosphotungstic acid left in solution, when the bases are precipitated, varies somewhat with the amount of the latter, so that the conditions of precipitation are not absolutely constant. It does not appear, however, that the variation is sufficient to cause significant change in the solubilities of the bases." Just how significant the changes in the solubilities of the bases are when different amounts of bases are present has never been shown. While the solubility corrections are comparatively small in magnitude, they may be quite large in comparison when the bases are present in small amounts, as is often the case. The average solubility corrections, expressed as percentages of the average uncorrected amino

acid nitrogen, for the six proteins analyzed by Van Slyke [9] are 122 per cent for cystine, 6.3 per cent for arginine, 21 for histidine, and 4.3 for lysine.

Whenever empirically determined correction factors reach the relative magnitude shown for cystine and histidine above, their use in a quantitative determination is questionable.

Errors. Of the four amino acids which the Van Slyke method is capable of determining, two, arginine and cystine, are determined directly and the other two, histidine and lysine, indirectly. Since the Van Slyke method is so widely used and commonly used with the idea that it gives quantitative results, a number of factors which may influence the accuracy of these determinations will be discussed.

(*a*) The determination of arginine. The direct estimation of arginine by boiling with alkali has been shown to be accurate,[142] although, when cystine is present to any considerable extent, the ammonia contributed by this acid must be considered.

(*b*) The determination of cystine. Cystine is calculated from the sulfur content of the solution of the bases. The method used for the estimation of the sulfur is quite accurate but cystine is readily decomposed by boiling with strong acids as well as by alkalies. Van Slyke [9] showed that as much as 50 per cent of cystine may not be recovered in the phosphotungstic acid precipitate after boiling for 24 hours with hydrochloric acid. Mörner [147] as well as Hofmann and Gortner [148] have shown that the properties of cystine are readily altered by boiling with strong acids. Gortner and Sandstrom [149] found that, "The cystine value of a Van Slyke analysis on a 24-hour hydrolysate may be taken to represent approximately 65 per cent of the true cystine nitrogen present in the unboiled material." The accuracy with which the cystine is determined indirectly influences the accuracy with which the histidine and lysine may be calculated for cystine evolves 107 per cent gas in the determination of amino nitrogen and a corresponding reduction in volume of gas is necessary.

(*c*) The determination of histidine and lysine. Since arginine contains three-fourths and histidine two-thirds of their respective total nitrogen in non-amino form and since it is assumed that there are no other amino acids in the basic fraction containing nitrogen in this form, the histidine may be calculated from the non-amino and arginine nitrogen content of the bases. The non-amino nitrogen content of the bases is the difference between the total nitrogen content, as determined by

[147] Mörner, K. A. H., *Z. physiol. Chem.*, 1901-2, xxxiv, 207.
[148] Hoffmann, W. F., and Gortner, R. A., *J. Am. Chem. Soc.*, 1925, xlvii, 1663.
[149] Gortner, R. A., and Sandstrom, W. M., *J. Am. Chem. Soc.*, 1925, xlvii, 1663.

the Kjeldahl method, and the amino nitrogen content, as determined by the nitrous acid method. The accuracy of the histidine and lysine determinations, therefore, depends upon the accuracy with which the total and the amino nitrogen of the bases can be determined. Some factors which may influence the accuracy of these determinations and thus the accuracy of the histidine and lysine values are the following:

(i) The action of nitrous acid upon the hexone bases. Van Slyke [132] stated that the α-amino groups of all the amino acids reacted with nitrous acid quantitatively in 5 minutes. The ε-amino group of lysine, however, required half an hour at 20°. In a subsequent paper [135] Van Slyke indicated that all the nitrogen of lysine was liberated in 15 minutes at 24°. Ordinarily, however, the bases are allowed to react with nitrous acid for half an hour. In this time it is assumed that lysine loses all of its nitrogen, histidine one-third, and arginine one-fourth.

Sure and Hart [150] investigated the effect of temperature on the reaction of lysine with nitrous acid and stated that at temperatures of 30°, or above, 10 or a maximum of 15 minutes would be more than sufficient for shaking the hexone bases at any concentration in protein analysis by the Van Slyke method. They also stated, without taking into consideration the time element, that at temperatures of 1° or below, the ε-amino group of lysine may become entirely inactive. Dunn and Schmidt,[151] however, showed that, contrary to the statement of Sure and Hart, lowering of the temperature decreased the rate with which the α-amino group (of alanine), as well as the ε-amino group of lysine, reacted with nitrous acid. They also showed that at temperatures as low as 4° all the nitrogen of lysine was evolved on treatment with nitrous acid for 50 minutes.

Plimmer [152] studied the action of nitrous acid upon the hexone bases and stated that lysine requires one hour for complete reaction at 14 to 17°. During a reaction time of one hour histidine evolves only one-third of its nitrogen but arginine evolves slightly more than one-fourth of its nitrogen; however, this introduced no appreciable error. That the guanidine group of arginine is slowly attacked by nitrous acid is further shown by Sekine,[153] who obtained one-half of the total nitrogen of arginine by treatment with nitrous acid for 2 or 3 hours at 20°. It is probable, however, notwithstanding these apparently anomalous findings, that, on treating the solution of the bases with nitrous acid for 30 minutes at 20°, as recommended by Van Slyke, lysine will evolve

[150] Sure, B., and Hart, E. B., *J. Biol. Chem.*, 1917, xxxi, 527.
[151] Dunn, M. S., and Schmidt, C. L. A., *J. Biol. Chem.*, 1922, liii, 401.
[152] Plimmer, R. H. A., *Biochem. J.*, 1924, xviii, 105.
[153] Sekine, H., Nogakukaiho, Tokio, 1919, i, 197; cited through *Chem. Abstr.*, 1921, xv, 243.

all of its nitrogen, histidine one-third, and arginine one-fourth, to a good degree of accuracy. Extreme variations in temperature, however, should not be permitted without corresponding changes in the time of reaction.

(ii) Errors involved in the phosphotungstic acid precipitation. The difficulties of quantitatively separating the monoamino from the diamino acids by phosphotungstic acid and the composition of the precipitate have been discussed above under the Hausmann method. That tryptophane forms a comparatively insoluble phosphotungstate under the conditions of the Van Slyke procedure was recognized by Van Slyke, but because this amino acid is so readily changed during hydrolysis into compounds not precipitated by phosphotungstic acid, it was not likely to be present. Concerning this, Van Slyke [9] says, "It appears improbable that tryptophane affects the composition of the phosphotungstic precipitate under the usual conditions of analysis, but it is advisable in the latter, as a precaution, to test a few drops of the solution of the bases for tryptophane."

Holm and Gortner,[154] however, found that in the presence of tryptophane the histidine values especially are higher than theoretical, and Gortner and Sandstrom [149] show that cystine, as well as histidine, is affected. The latter authors show also, contrary to the statements of Van Slyke, that proline forms a rather insoluble phosphotungstate and when present in a protein causes amounts of basic nitrogen far in excess of that actually present. In this case the histidine value and also, because of its entire lack of amino nitrogen, the lysine value are affected.

The influence of the solubility of the bases and the possibility of inclusion or occlusion of monoamino acids is recognized by Van Slyke in the careful control of conditions for precipitation, filtration, and washing of the precipitate, and in the application of solubility corrections.

Plimmer and Rosedale,[155] in a comparative series of determinations of the nitrogen distribution in proteins, state that the discrepancies often found in the analysis of the same protein are connected mainly with the incomplete precipitation of the hexone bases with phosphotungstic acid. The greatest differences were found in the amino nitrogen determinations. These authors suggest that more concordant results will be obtained if the total, as well as the amino, nitrogen, be not recorded beyond tenths of a milligram.*

[154] Holm, G. E., and Gortner, R. A., *J. Am. Chem. Soc.*, 1920, xlii, 2378.
[155] Plimmer, R. H. A., and Rosedale, J. L., *Biochem. J.*, 1925, xix, 1004.

* Gortner and Hoffmann (*J. Am. Chem. Soc.*, 1925, xlvii, 580) claimed that the temperature at which the phosphotungstic acid precipitation was carried out markedly affected the results obtained. While this was not considered as a significant source of error by Plimmer and Rosedale, Kernot and Knaggs (*Biochem. J.*, 1928, xxii, 528) have recently obtained data which indicate a greater influence of temperature upon the nitrogen precipitated by phos-

(iii) Abnormal behavior of cystine. The fact that only a fraction of the original cystine present is recovered in the solution of the bases does not affect the accuracy of the determinations of arginine, histidine, and lysine. The arginine determination, however, may be affected if an accurate estimation of the amount of cystine in the solution of the bases is not obtained, because cystine evolves approximately 18 per cent of its nitrogen as ammonia nitrogen under the conditions of the arginine determination. This correction, however, is negligible in all cases except the keratins. Cystine also behaves abnormally towards nitrous acid in that it evolves 107 per cent of the theoretical amount of nitrogen gas. The amino nitrogen determination of the bases must be correspondingly reduced whenever the cystine value is significant.

(*d*) Factors influencing the accuracy of the monoamino and the non-amino nitrogen in the filtrate from the basis. The abnormal behavior of cystine, tryptophane, proline, and glycine all influence the accuracy of the monoamino and the non-amino nitrogen values in the filtrate from the bases. Cystine and tryptophane, due to partial decomposition into unknown products during hydrolysis with acids, cause an increase in the ammonia fraction with a corresponding decrease in monoamino nitrogen.[149] Proline and tryptophane, being partially precipitated along with the bases, influence the monoamino and non-amino nitrogen content of the filtrate as well as the basic fractions.[149] Glycine gives more than the theoretical amount (103%) of nitrogen when treated with nitrous acid.[132] Inasmuch as the bases are all soluble to a slight extent, the filtrate fractions are correspondingly increased.*

Comparison of the Van Slyke Method with the Kossel and Patten Method

Because of the rather wide variations for the values of cystine, arginine, histidine, and lysine in casein obtained by Van Slyke by his nitrogen distribution method at different times,[81, 146] he compared [146] the nitrogen distribution method with the Kossel and Patten method, as modified by Osborne, Leavenworth, and Brautlecht.[2] In general the values for the diamino acids are higher by the Van Slyke method than by the Kossel and Patten method. The agreement is closest in case of arginine. Van Slyke [146] has this to say concerning the two methods: "From the data in this and previous papers,[9] which permit a comparison

photungstic acid and upon the solubilities of the phosphotungstates than has previously been supposed.

* A critical study of the Van Slyke method has been recently published by W. L. Davies (*Biochem. J.*, 1927, xxi, 814). It has been concluded by Davies that the phosphotungstic acid precipitation of the bases is less complete than Van Slyke has supposed, and that the determination of the amino nitrogen of the precipitated bases is also incomplete. Certain modifications of the method are suggested to avoid these errors.

of results by the two methods, it appears that the nitrogen distribution method is somewhat more reliable than the Kossel method for lysine determination in proteins, that both methods are quite accurate for arginine, and that the Kossel-Patten method, as modified by Osborne, Leavenworth, and Brautlecht, gives more consistent results for histidine." A further comparison between the two methods was made by Osborne, Van Slyke, Leavenworth and Vinograd,[77] the results of which agree with those of Van Slyke just mentioned.

Concerning the accuracy of the Kossel and Patten method, Osborne, Leavenworth and Brautlecht [2] have shown rather conclusively that the method, while tedious, is capable of an excellent degree of accuracy; and Vickery [6] says, "Although the method introduced by Kossel and Kutscher for estimating the basic amino acids in proteins leaves little to be desired on the score of accuracy, there is room for much improvement in convenience."

Comparison of the Van Slyke and Sörensen Methods for Amino Nitrogen Determinations

These two methods have been compared repeatedly on protein hydrolysates. White and Thomas [156] found that the two methods gave parallel, but the Van Slyke method the lower, results. Rogosiński [157] and Anderson [158] both concluded that the Sörensen method gave the more satisfactory results. Abderhalden and Kramm [159] found that the Van Slyke method gave results after a 5-minute period that were lower than were given by the Sörensen method, but after a 10-minute period the Van Slyke results were higher. These authors suggested that the Sörensen method should be used to standardize the reaction time in the Van Slyke procedure. Northrop [160] concluded that the Van Slyke method gave the more accurate results for the absolute determinations of the amino acids, but that for comparative experiments involving the study of the changes occurring in gelatin solutions, the formol titration method was more accurate and more rapid.

Wilson [161] compared the Van Slyke and the Sörensen methods for the estimation of the free amino nitrogen in native and derived proteins. He concluded that the Van Slyke method gave results which depended upon the reaction time and upon the type of material examined. He believed also that the high results were due to hydrolysis of the protein

[156] White, G. F., and Thomas, A., *J. Biol. Chem.*, 1912-13, xiii, 111.
[157] Rogozinski, F., *Z. physiol. Chem.*, 1912, lxxix, 398.
[158] Anderson, A. C., *Biochem. Z.*, 1915, lxx, 344.
[159] Abderhalden, E., and Kramm, F., *Z. physiol. Chem.*, 1912, lxxvii, 425.
[160] Northrop, J. H., *J. Gen. Physiol.*, 1920-21, iii, 715.
[161] Wilson, D. W., *J. Biol. Chem.*, 1923, lvi, 191.

and low results could be caused by the precipitation of the protein by the reagents. For this type of determination he favored the Sörensen method in that it is less susceptible to errors of this nature. The Sörensen method, however, is not free from theoretical errors.

Use of the Van Slyke Method. The possibility of errors in the Van Slyke procedure and the comparisons with other methods all testify to the inadequacy of our methods for studying the composition of proteins. However, Van Slyke's method for determining the free amino nitrogen [132] and his nitrogen distribution method for protein analysis [9] have been widely applied.

Hartley [141] used the method in studying proteins of ox and horse serum. Crowther and Raistrick [162] used the method in the analysis of proteins of colostrum and milk and Drummond [163] applied it to a study of normal human and chicken tissues for comparison with pathological human tissues with special reference to cancer.

The method has been used very generally in studies of nitrogen distribution in proteins of foods, feeds and plants. It has been applied directly to the analysis of feeds without the isolation of the different proteins. Such analyses have been made by Grindley, Joseph and Slater,[164] Grindley and Slater,[51] Nollau,[165] Brewster and Alsberg,[165a] Nevens,[166] Hamilton, Nevens and Grindley,[167] and by Hamilton, Uyei, Baker and Grindley.[168] Van Slyke emphasized the fact that his method could not be expected to give accurate results except in the analysis of pure proteins and Hart and Sure,[169] Gortner,[49] and Osborne, Van Slyke, Leavenworth and Vinograd [77] have shown that the presence of carbohydrates interferes to a considerable extent in the application of the method. In the analyses by Nevens, by Hamilton, Nevens, and Grindley, and by Hamilton, Uyei, Baker and Grindley, the carbohydrates were fairly completely removed by extraction before the application of the Van Slyke method and the authors felt that, while concordant results were difficult to obtain, the results were of considerable value in comparing the nutritive value of the combined proteins in feeds. It is doubtful, however, whether the labor involved in such analyses is warranted, especially in view of the meagerness of results finally obtained by the Van Slyke procedure.

[162] Crowther, C., and Raistrick, H., *Biochem. J.*, 1916, x, 434.
[163] Drummond, J. C., *Biochem. J.*, 1916, x, 473.
[164] Grindley, H. S., Joseph, W. E., and Slater, M. E., *J. Am. Chem. Soc.*, 1915, xxxvii, 1778.
[165] Nollau, E. H., *J. Biol. Chem.*, 1915, xxi, 611.
[165a] Brewster, J. E., and Alsberg, C. A., *J. Biol. Chem.*, 1919, xxxvii, 367.
[166] Nevens, W. B., *J. Dairy Sci.*, 1921, iv, 375.
[167] Hamilton, T. S., Nevens, W. B., and Grindley, H. S., *J. Biol. Chem.*, 1921, xlviii, 249.
[168] Hamilton, T. S., Uyei, N., Baker, J. B., and Grindley, H. S., *J. Am. Chem. Soc.*, 1923, xlv, 815.
[169] Hart, E. B., and Sure, B., *J. Biol. Chem.*, 1916, xxviii, 241.

Kober's Copper Method

Proteins and their degradation products, peptones, peptides, and amino acids, form complexes with many of the heavy metals. Of these complexes, those of amino acids and the heavy metals are best known. Kober [170, 171] and Kober and Sugiura [172] proposed a method for the quantitative determination of amino acids based upon the property of these acids of forming definite compounds with copper.

The principle of the method is to form, from the amino acids and copper carbonate or hydroxide, definitely constituted copper salts, from which the copper may be nearly quantitatively precipitated as copper oxide by boiling with sodium hydroxide. The copper oxide is then removed by filtration and, from a determination of the copper present, the quantity of amino acids may be estimated. Peptones and peptides form similar copper complexes, but the copper from these is but little precipitated by boiling alkali.

The general formula for the copper complexes is CuA_2, where A is a monobasic α-amino acid. In case of dibasic amino acids the formula is CuA. The recovery of copper averages 99 per cent of the amount computed on the basis of these formulæ, histidine only being an exception in yielding a low value of 94.9 per cent. In order to recover completely the copper it is necessary to determine the copper in both the precipitate and in the filtrate, since the precipitation is not exactly quantitative. It is claimed that under the conditions outlined by the method a small but definite fraction remains unprecipitated. This fraction is 13.5 per cent of the total copper.

The method is applicable to the determination of the amount of free amino acids in a protein hydrolysate and in following the rate of hydrolysis. Kober and Sugiura [172] have developed the method into a micro-chemical method applicable to the estimation of amino acids in blood and urine.

While the authors have compared their method quite favorably with the ninhydrin, the Sörensen, and the Van Slyke methods, the method has not been widely used, probably because of its lack of specificity for α-amino acids alone.

Electrolytic Method

While electrolysis has not as yet been used as a part in any quantitative method for the estimation of the amino acids, its use in the separation of certain groups of these acids is of especial consideration in this

[170] Kober, P. A., *J. Biol. Chem.*, 1911, x, 9.
[171] Kober, P. A., *J. Ind. Eng. Chem.*, 1917, ix, 501.
[172] Kober, P. A., and Sugiura, K., *J. Amer. Chem. Soc.*, 1913, xxxv, 1546.

connection. In 1912 Ikeda and Suzuki [173] patented a process for separating glutamic acid from a protein hydrolysate by electrolysis. They made the observation that on passing a direct current through a solution containing the products of protein hydrolysis placed in the center of a three-compartment cell, the amino acids soon separated into three fractions consisting of (*a*) the amino acids which are predominantly acid, including aspartic and glutamic acids, which migrate to the anode, (*b*) the basic amino acids, arginine, histidine, and lysine, which migrate to the cathode, and (*c*) the remaining amino acids, which on account of the fact that their acid properties are nearly balanced by their basic properties, remain in the center compartment.

Foster and Schmidt [174, 175] have made use of this idea in the separation of the hexone bases from a protein hydrolysate. By a second electrolysis of the basic fraction under proper conditions of acidity they were able to separate histidine from the other two bases almost quantitatively. This method has been used by the authors for the preparation of pure arginine which was precipitated as the picrolonate.

The use of electrolysis in separating different groups of the amino acids preparatory to their isolation or determination seems very promising and if it can be placed on a quantitative basis one of the most laborious and expensive operations in protein analysis will be eliminated.

Foreman's, Harris', and Other Volumetric Methods

In 1920 Foreman [176] described a volumetric method for the estimation of free amino acids based upon an observation by Sutton [177] that the acid in an ammonium salt could be titrated with standard alkali in alcoholic solution using phenolphthalein as the indicator. Foreman found that when aqueous-alcoholic solutions of certain amino acids containing about 85 per cent alcohol were titrated with standard alcoholic potash, the amino or imino groups resembled ammonia in showing no basicity to phenolphthalein, and the carboxyl groups could be accurately estimated. All the amino acids with the exception of aspartic and glutamic acids, proline and arginine may be quantitatively determined by titration of a neutral 85 per cent alcoholic solution with standard alcoholic potash using phenolphthalein as indicator. By the subsequent addition of formalin, as in the Sörensen method,[29] or acetone, and continued titration, the amount of aspartic and glutamic acids and proline may be quantitatively measured. Arginine was the only amino acid investigated which could not be

[173] Ikeda, K., and Suzuki, S., U. S. Patent No. 1,015,891, Jan. 30, 1912.
[174] Foster, G. L., and Schmidt, C. L. A., *Proc. Soc. Exp. Biol. Med.*, 1921-22, xix, 348.
[175] Foster, G. L., and Schmidt, C. L. A., *J. Biol. Chem.*, 1923, lvi, 545.
[176] Foreman, F. W., *Biochem. J.*, 1920, xiv, 451.
[177] Sutton, F., "Volumetric Analysis," 8th edition, p. 38; cited through Foreman.[176]

determined by this method. For total amino acids the titration after the addition of formaldehyde is more nearly quantitative than a combination of the titrations before and after the addition of formaldehyde, since glycine and possibly other amino acids gave low results when titrated in the absence of the aldehyde. The method is claimed to be suitable to the analysis of such organic mixtures as result from the degradation of proteins or similar bodies by acids, enzymes, or bacteria.

A method similar to the Foreman method has been described also by Willstätter and Waldschmidt-Leitz.[178] According to these investigators, the titration of amino acids is to be carried out in 97 per cent alcohol; polypeptides can be titrated in 40 per cent alcohol. By titrating first in 40 per cent alcohol and then in 97 per cent alcohol an estimate can be made of both total polypeptides and total amino acids in a mixture.

Harris [179] has given an excellent discussion of the theoretical considerations involved in the titration of the amino and carboxyl groups of the amino acids. In the opinion of Harris, the explanation usually offered, that the original amino acid is neutral because the basic amino group neutralizes the acidic carboxyl group, and that with the addition of formaldehyde the basic character of the amino group is destroyed with the result that the acidic carboxyl group is free to be titrated, is not entirely correct. He explains the neutrality of the amino acids by the fact that neither the amino nor the carboxyl groups are appreciably ionized in neutral solution. That the carboxyl group can be titrated after the addition of formaldehyde or alcohol or acetone, as in the Sörensen and Foreman methods, depends upon the fact that these solvents cause an increase ($ca \times 1000$) in the value of the acid ionization constant above that in aqueous solution.

Harris found that those amino acids which give a low reading when titrated in 85 per cent alcohol according to the Foreman method titrate quantitatively when phenolphthalein is substituted by thymolphthalein, an indicator possessing a more alkaline transition point. With this as the basis for modifying Foreman's method, Harris described a new titration method in alcohol for the determination of the amino acids. Using thymolphthalein in place of phenolphthalein a smaller concentration of alcohol is sufficient to obtain quantitative results, and aqueous soda may replace alcoholic potash. Further, using thymolphthalein there is a sharper end-point color change.

Harris also investigated the action of various solvents on the amino acids in an endeavor to produce a rise in K_b for the amino groups,

[178] Willstätter, R., and Waldschmidt-Leitz, E., *Ber. chem. Ges.*, 1921, liv (2), 2988.
[179] Harris, L. J., *Proc. Roy. Soc.* (*London*), 1923-24, B, xcv, 500.

similar to the rise in K_a for the carboxyl group produced by the presence of alcohol or acetone. Could such a solvent be found, the amino group could be completely titrated with standard acid at a much less acid end-point than is necessary in aqueous solution, and the attainment of the end-point would be shown by the indicator undergoing a sharp change. Although the search did not lead to the discovery of a suitable solvent, Harris evolved a simple method which may be used in combination with his modified Foreman method for the estimation of amino as well as carboxyl groups in an amino acid mixture. The method is briefly as follows:

The carboxyl groups are first estimated by the addition of alcohol (80 per cent of the final volume) and titrating with $N/10$ soda until a blue color appears, using thymolphthalein as the indicator. In the presence of strong acid or alkali this titration is a measure of the carboxyl groups plus strong acid or minus alkali.

To the same solution methyl-red is added and the solution titrated to an orange color with $N/10$ hydrochloric acid. The amount of hydrochloric acid required is equivalent to the total amino groups present. The carboxyl and one amino group in arginine do not titrate. A separate blank correction should be applied to each of the two titrations: (*a*) the amount of $N/10$ alkali required by the water-alcohol-thymolphthalein mixture when no amino acid is present, and (*b*) the amount of $N/10$ hydrochloric acid required in the back titration of the same mixture to methyl-red.

In a previous paper [180] Harris discussed the applicability of volumetric methods, based on the theory of titration, to the examination of amino acid mixtures. He showed, for example, that by determining the complete titration curve of any mixture of amino acids, an estimation could be deduced of each of the following groups: monoamino-monocarboxylic acids, dicarboxylic-monoamino acids, and diamino-monocarboxylic acids. The use of the quinone electrode is suggested,[180, 181] as a convenient and accurate method for determining amino or other basic or acidic groups in an amino acid mixture. He showed that with a knowledge of the percentage ionization of various acidic and basic groups at given pH values, it is possible to estimate accurately such groups by titrating to the given pH values in the presence of suitable indicators. The following are the types of estimations possible: (*a*) a single amino acid (alone or in the presence of mineral acid or alkali), (*b*) total amino groups, and each of the following, alone or in the

[180] Harris, L. J., *Proc. Roy. Soc. (London)*, 1923-24, B, xcv, 440.
[181] Harris, L. J., *J. Chem. Soc.*, 1923, cxxiii, 3294.

presence of a mixture of monoamino-monocarboxylic acids, (*c*) glutamic acid, (*d*) lysine, and (*e*) histidine.

Similar volumetric methods for the estimation of the amino acids are the electrometric titration methods of Tague [182] and of Widmark and Larson.[183] Hirsch [184] has also proposed a volumetric method for the estimation of the amino acids.

The development of the modern theory of titration and its application to analytical operations will undoubtedly play an important part in the future in the development of methods for the estimation and preparation of the amino acids.

Dakin's Butyl Alcohol Extraction Method

In 1918 Dakin [3] found that by long continued extraction of a protein hydrolysate with partially miscible solvents, such as butyl alcohol, the feebly ionized monoamino acids and proline were nearly quantitatively removed, while the strong dicarboxylic and diamino acids remained behind. By the use of butyl alcohol as the solvent the products of protein hydrolysis may be, according to Dakin, readily separated almost completely into the following five groups:

(1) Monoamino acids, both aliphatic and aromatic, insoluble in alcohol but extracted by butyl alcohol.

(2) Proline, soluble in alcohol and extracted by butyl alcohol.

(3) Peptide anhydrides (diketopiperazines) extracted by butyl alcohol, but separated from (2) by sparing solubility in alcohol or water.

(4) Dicarboxylic acids, not extracted by butyl alcohol.

(5) Diamino acids, not extracted by butyl alcohol, but separable from (4) by phosphotungstic acid and other means.

The individual amino acids in each of the above groups may then be estimated by suitable means. The method may be used very advantageously as a supplement to the Fischer esterification, the Kossel and many other methods for the determination, isolation and preparation of groups of or of individual amino acids. Dakin's procedure represents a most important advance in the technic of protein analysis. By this means the products of a protein hydrolysate may be divided without serious loss into five groups composed of chemically similar individuals and each group may be readily obtained in solid form. The process causes no racemization; thus the amino acids possess their full optical rotation. The method should therefore be valuable for the purpose of

[182] Tague, E. L., *J. Amer. Chem. Soc.*, 1920, xlii, 173.
[183] Widmark, E. M. P., and Larson, E. L., *Biochem. Z.*, 1923, cxl, 284.
[184] Hirsch, P., *Biochem. Z.*, 1924, cxlvii, 433.

preparing the natural forms of the amino acids for chemical study or for bacterial and animal metabolism experiments.

Materially higher yields of many amino acids were obtained than was possible by previous methods. Dakin was able to increase the summation of amino acids in a protein to unprecedented figures. He recovered, as individual products, 91.3 per cent of the gelatin molecule [4] and 100 per cent of zein.[5]

In Dakin's original method,[3] the extraction was conducted in a Steudel apparatus at atmospheric pressure, whereby the amino acids were subjected to the action of boiling butyl alcohol for long periods of time. To this latter fact Dakin attributed the difficulty which he encountered in the purification of proline, which is extracted from the aqueous solution and remains dissolved in the alcohol. To avoid this objection Dakin later [4] conducted the extraction *in vacuo*. Proline was readily extracted under these conditions and could be obtained in a satisfactorily pure condition, but the yield and rate of extraction were but little improved. Osborne, Leavenworth and Nolan [185] found that the same result could be effected and the method applied on a large scale by dropping the solution of the amino acids at room temperature into a tall jar containing a large amount of butyl alcohol, which was rapidly stirred at the same time. By distilling off the butyl alcohol *in vacuo* the amino acids need not be heated with the alcohol for more than a short time and then only at a low temperature. Even by this method, however, the proline fraction is not free from diketopiperazines and consequently Dakin's method cannot be used to estimate the proline unless the former are hydrolyzed, or the proline esterified.

It was in connection with investigations concerned with the extraction of amino acids by means of partially miscible solvents that Dakin,[3, 186] using Foreman's method [187] for the determination of the dibasic amino acids, discovered that β-hydroxyglutamic acid was a normal constituent of certain proteins.

Folin's Colorimetric Method

Another colorimetric method for the quantitative estimation of the amino acids was proposed by Otto Folin [188] in 1922. The method depends upon the production of a red color when the amino acids are treated with β-naphthoquinone-sulfonic acid. The color developed by the amino acids and the quinone reagent under carefully controlled

[185] Osborne, T. B., Leavenworth, C. S., and Nolan, L. S., *J. Biol. Chem.*, 1924, lxi, 309.
[186] Dakin, H. D., *Biochem. J.*, 1919, xiii, 398.
[187] Foreman, F. W., *Biochem. J.*, 1914, viii, 463.
[188] Folin, O., *J. Biol. Chem.*, 1922, li, 377, 393.

conditions is matched in a colorimeter against a standard prepared from a pure amino acid, usually glycine.

β-Naphthoquinone-sulfonic acid does not give a color with any of the main nitrogenous waste products except ammonia, and this is easily removed. Urea, uric acid, creatinine, creatine, or hippuric acid produce no color with the reagent. Indole produces a blue color, but unless this substance is present in excessive amounts, the color is not developed under the conditions used for the production of the color with the amino acids.

While the method was developed primarily for the estimation of amino acids in blood and urine, it also may be adapted for determination of these acids in other materials, such as gastric contents, milk, meat extracts, protein hydrolysates, etc.

Other Methods

There have been proposed, for the estimation of amino acids, a number of methods which have not as yet found wide application. While no method yet proposed for the quantitative determination of the amino acids has proved entirely satsifactory, some few of the older methods have perhaps been used too exclusively. All new methods, and perhaps some of the lesser known older methods, should be studied carefully in the hope that a more specific and more satisfactory method for the quantitative estimation of the amino acids might be found.

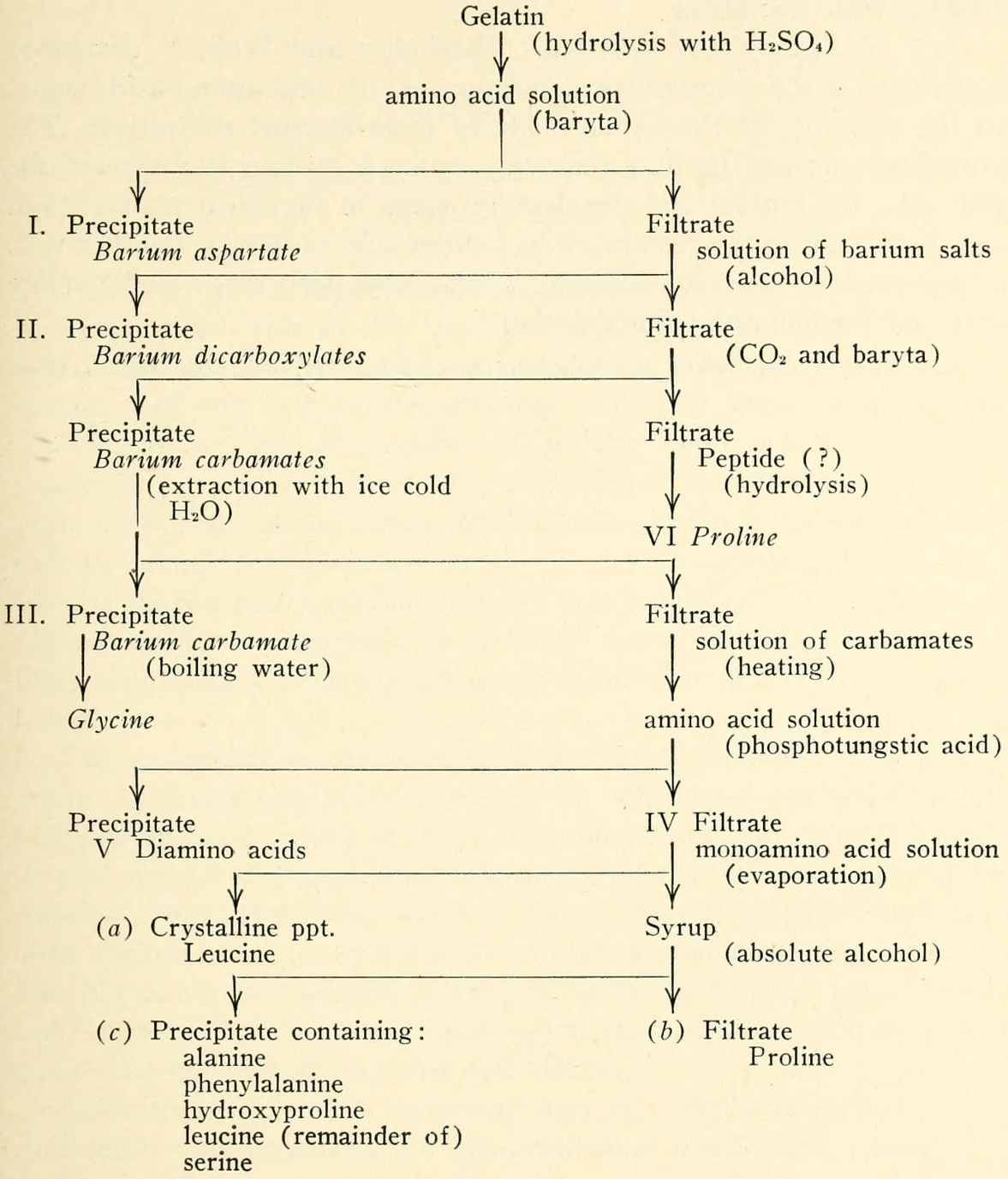
(*a*) *The Carbamate Method of Kingston and Schryver.* In 1924 Kingston and Schryver [189] described a method for the isolation and determination of the amino acids, based on the ability of the amino acids to form insoluble carbamates, a class of compounds originally described by Siegfried, when a solution containing them is treated in the presence of alcohol with barium hydroxide and carbon dioxide. The general scheme of separation is outlined in the diagram on page 139.

The separation of the amino acids in the last fraction containing alanine, phenylalanine, hydroxyproline, leucine, and serine was the least satisfactory, although by summation of all products 93 per cent of the gelatin molecule was accounted for.

(*b*) *A New Gasometric Method by Ashmarin.* Ashmarin [190] has proposed, for the quantitative determination of the carboxyl groups in a protein hydrolysate, a method similar to the Sörensen method except, instead of titrating the carboxyls, an excess of potassium acid carbonate is added and the carbon dioxide liberated is indirectly

[189] Kingston, H. L., and Schryver, S. B., *Biochem. J.*, 1924, xviii, 1070.
[190] Ashmarin, P. A., (Russ.) *Arch. Biol. Sci.*, 1924, xxiii, 347; through *Chem. Abst.*, 1924, xviii, 2536.

Diagram of the Separation of the Hydrolysis Products of Gelatin.
(*Taken from Kingston and Schryver.*)



measured by weighing the amount of mercury displaced from the system. Each molecule of carbon dioxide corresponds to one carboxyl group. The advantages of the method, according to the author, are: the direct estimation of the carboxyl groups; the freedom from the subjective error in judging the end point of a titration with an indicator; the accuracy of the determination is not affected by turbidity or color of a solution; the quantity of water in the reaction mixture may be reduced to the amount contained in the formalin; the reaction takes

place and is completed at a constant hydrogen ion concentration; and the potassium acid carbonate is less apt to cause further hydrolysis than titration with free alkali.

(*c*) *A Benzoylation Method.* Cherbuliez and Wahl [191] developed a method for the quantitative determination of total amino acids based on the property of the amino acids to form benzoyl derivatives. The procedure consists in first removing from a protein hydrolysate the ammonia, the humin, and the diamino acids in the usual manner, and then benzoylating the remainder in sodium acid carbonate solution with a large excess of benzoyl chloride. The benzoyl derivatives of the amino acids are precipitated by acidulation.

[191] Cherbuliez, E., and Wahl, R., *Helvetica Chim. Acta*, 1925, viii, 571; through *Chem. Abstr.*, 1926, xx, 59.

CHAPTER III

THE DETERMINATION OF THE INDIVIDUAL AMINO ACIDS

As stated in the beginning of the previous chapter, the composition of proteins in terms of the constituent amino acids is a problem that is still far from solution. Methods are available for the accurate determination of but few of the nineteen definitely known amino acids. The reason for this is obvious. All of the amino acids are more or less similar in constitution and therefore similar in chemical and physical properties. Some group similarities, however, make it possible to effect a fairly complete division into somewhat smaller groups, but the members of these smaller groups resemble each other still more closely and consequently their separation is made still more difficult. It is, therefore, extremely difficult to find reactions which are specific for any one amino acid.

Fischer introduced the method of fractional distillation of the ethyl esters under extremely low pressures in order to prepare the various amino acids for purposes of study and it has been through modifications of this method that most of our knowledge concerning the monoamino acids of proteins has been obtained. Other derivatives which have been most useful in the determinations of the amino acids are the silver salts, the phosphotungstates, and especially the flavianates, which Kossel and Gross, and Vickery and Leavenworth have used to such advantage in the determination of arginine and histidine.

Derivatives as a rule, however, are not readily adaptable to the quantitative determination of the individual amino acids, since the derivative of one amino acid is usually so nearly alike, in solubilities and other physical properties, to the same derivative of another amino acid that the two cannot be completely separated. It is, therefore, necessary that, in the determination of an individual amino acid, any chemical groups or peculiar configurations specific to that amino acid be made use of. For example, reactions characteristic of the phenolic configuration in tyrosine, the indole group in tryptophane, the guanidine group in arginine, the sulfur in cystine, the imidazole group in histidine, etc., have all been used in the determination of these amino acids.

The methods to be described below are methods for the estimation, usually of a single amino acid. The methods proposed for the determination of more than one amino acid, or of a group of amino acids have been described in the preceding chapter.

By the use of all methods available, only half of the nineteen known amino acids can be determined with any degree of accuracy. Arginine, histidine, and lysine may, perhaps, be determined with the greatest degree of accuracy. Methods for the determinations of cystine, tyrosine, tryptophane, glutamic acid, and proline have also been developed until the respective amounts of each of these amino acids in a protein may be determined with a fair degree of accuracy. The principles involved in each of these methods will now be discussed.

TYROSINE

Gravimetric Determination

Tyrosine, on account of its extremely slight solubility in neutral aqueous solutions, has been determined in protein hydrolysates simply by neutralizing, concentrating, and allowing the solution to stand. In the older methods the protein was hydrolyzed with sulfuric acid, which was quantitatively removed with barium. The filtrate was evaporated to a small volume and allowed to stand. Tyrosine was crystallized out and the crystals removed. The mother liquors were further evaporated and additional crystals collected, the processes of evaporation and crystallization being repeated until the solution no longer gave Millon's test for tyrosine. The tyrosine may be recrystallized from water. The yield of tyrosine thus obtained is a measure of its amount in the protein.

On account of the difficulty of filtering and completely washing the barium sulfate precipitate, Abderhalden and Teruuchi [1] hydrolyzed silk with hydrochloric acid. As much of the hydrochloric acid as possible was removed by repeated concentrations *in vacuo*. From a small aliquot the hydrochloric acid remaining was then determined and the main bulk exactly neutralized by the addition of the calculated quantity of sodium hydroxide. The tyrosine was then allowed to crystallize in the same manner as when sulfuric acid had been used. Later Abderhalden [2] shortened the process by hydrolyzing with hydrochloric acid, removing most of the acid by repeated evaporations *in vacuo,* finally dissolving the residue in ammonia, and again evaporating *in vacuo* to dryness. On taking up the residue with cold water the tyrosine remains. Plimmer [3, p. 18] recommends the use of hot water so that the solution may

[1] Abderhalden, E., and Teruuchi, Y., *Z. physiol. Chem.,* 1906, xlviii, 528.
[2] Abderhalden, E., *Z. physiol. Chem.,* 1912, lxxvii, 75.
[3] Plimmer, R. H. A., "The Chemical Constitution of the Proteins," Part I, 1917.

be decolorized with charcoal before allowing the tyrosine to crystallize.

For large quantities of protein, Plimmer [3] hydrolyzed with hydrochloric acid, removed most of the hydrochloric acid by concentration *in vacuo,* then removed most of the remaining acid by the addition of cuprous oxide. After filtering off the cuprous chloride, the excess copper was removed with hydrogen sulfide and the latter by a current of air. The remainder of the hydrochloric acid was then either neutralized by the calculated amount of soda or was precipitated as silver chloride. On concentration the tyrosine precipitated out.

Levene and Van Slyke [4] used hydrochloric acid as the hydrolytic agent, removed the greater part of it by concentration *in vacuo,* then most of the remaining acid with lead oxide. The precipitate of lead oxychloride retained the resinous matters and a nearly colorless filtrate resulted. The rest of the chlorine was removed by the addition of the calculated amount of silver sulfate, the excess lead by adding sulfuric acid and passing in hydrogen sulfide, and sulfuric acid by baryta. On concentrating the solution to one-seventh, almost pure tyrosine separated out. It was filtered off, washed, dried and weighed.

The solubilities of cystine are much like those of tyrosine, making the separation by crystallization difficult. In most cases the amount of cystine present is so small that its presence may be neglected in the determination of tyrosine by this method. In the case of proteins containing comparatively large amounts of cystine, *i.e.,* the keratins, Plimmer [5] separated these two compounds by esterification of the tyrosine by means of alcohol saturated with hydrochloric acid. The tyrosine is readily esterified and passes into solution, while the cystine is not. Tests showed that 94 to 100 per cent of the tyrosine might be recovered from mixtures by this process.

If the neutral solution is concentrated too far a mixture of tyrosine and leucine may separate. These two amino acids may be separated by dissolving the leucine in glacial acetic acid. Tyrosine is insoluble.[6]

In general it may be considered that the results obtained by the gravimetric method are minimal, for Folin and Denis,[7] Osborne and Clapp,[8] Osborne and Guest,[9] Abderhalden and Fuchs,[10] and others have

[4] Levene, P. A., and Van Slyke, D. D., *Biochem. Z.,* 1908, xiii, 440; *J. Biol. Chem.,* 1910, viii, 285.
[5] Plimmer, R. H. A., *Biochem. J.,* 1913, vii, 311.
[6] Habermann, J., and Ehrenfeld, R., *Z. physiol. Chem.,* 1902, xxxvii, 18.
[7] Folin, O., and Denis, W., *J. Biol. Chem.,* 1912, xii, 245.
[8] Osborne, T. B., and Clapp, S. H., *Am. J. Physiol.,* 1906, xvii, 231; 1907, xx, 475.
[9] Osborne, T. B., and Guest, H. H., *J. Biol. Chem.,* 1911, ix, 333.
[10] Abderhalden E., and Fuchs, D., *Z. physiol. Chem.,* 1913, lxxxiii, 468.

shown that it is extremely difficult to crystallize all of the tyrosine from any protein hydrolysate.

The Folin and Denis Colorimetric Method (1912)

Folin and Denis [7] proposed a colorimetric method for the quantitative estimation of tyrosine. The method is based upon the development of a blue color which phenols give with a phosphotungstic-phosphomolybdic acid reagent developed by them.[11] A small quantity of the protein is hydrolyzed and an aliquot of the hydrolysate is used for the development of the color which is compared in a Duboscq colorimeter with the color developed at the same time from a standard tyrosine solution. Compared with the gravimetric values for tyrosine, the values by the colorimetric method were always considerably higher. Folin and Denis tested all the then known amino acids with the exception of tryptophane and hydroxyproline and found that none besides tyrosine gave the color. They claimed that indole and indole derivatives did not interfere so that, in the absence of other phenols, the reaction was specific for tyrosine and that the values obtained by the colorimetric method represented more nearly than did the gravimetric method the correct values for tyrosine.

The colorimetric method of Folin and Denis has been adversely criticized by a number of different investigators, mainly on the basis of its lack of specificity for tyrosine. Abderhalden and Fuchs [10] showed that tryptophane also produced a blue color with the phenol reagent and that the results of Folin and Denis were probably too high due to this fact. That tryptophane did yield a blue color with the phenol reagent was partially recognized by Folin and Denis.[12] Later Gortner and Holm [13] as well as Thomas [14] verified the findings of Abderhalden and Fuchs and also showed that indole and its derivatives produced a blue color with the reagent.

Abderhalden [15] further stated that hydroxyproline gave the blue color with the tyrosine reagent, but Johns and Jones [16] showed that this could not be a significant error, since gelatin containing 6 per cent hydroxyproline gave after hydrolysis but little color with the reagent. Fürth and his collaborators [17, 18, 19] also criticized the Folin and Denis method on much the same grounds as did Gortner and Holm.

[11] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xii, 239.
[12] Folin, O., and Denis, W., *J. Biol. Chem.*, 1913, xiv, 457.
[13] Gortner, R. A., and Holm, G. E., *J. Am. Chem. Soc.*, 1920, xlii, 1678.
[14] Thomas, P., *Bull. soc. chim. biol.*, 1921, iii, 197.
[15] Abderhalden, E., *Z. physiol. Chem.*, 1913, lxxxv, 91.
[16] Johns, C. O., and Jones, D. B., *J. Biol. Chem.*, 1918, xxxvi, 319.
[17] Fürth, O., and Fleischmann, W., *Biochem. Z.*, 1922, cxxvii, 137.
[18] Fürth, O., *Biochem. Z.*, 1924, cxlvi, 259.
[19] Fürth, O., and Fischer, A., *Biochem. Z.*, 1924, cliv, 1.

The Folin and Looney Colorimetic Method (1922)

In 1922 Folin and Looney [20] acknowledged the errors caused by tryptophane and by indole and its derivatives in the Folin and Denis method and also stated that the tyrosine results obtained by this method were in many cases undoubtedly too high. The Folin and Denis procedure was modified, it was claimed, so as to eliminate the errors in the tyrosine determination due to tryptophane. At the same time procedures for the colorimetric estimations of tryptophane and of cystine were also described. In the modified procedure for tyrosine estimation the tryptophane was removed by precipitation with the mercuric sulfate reagent of Hopkins and Cole. By maintaining an acidity between 3.5 to 7.5 per cent sulfuric acid no tyrosine was removed and the tryptophane was quantitatively precipitated. Even with this procedure Folin and Looney obtained certain inconsistencies in the case of the tyrosine content of gliadin and of edestin. In 1926 Looney [21] redetermined the tyrosine content of gliadin and edestin as well as of several other proteins and at the same time answered much of the adverse criticism that had been directed against the Folin and Looney methods for the determination of tyrosine and tryptophane.

The Folin and Ciocalteu Method Based on Millon's Test (1927)

In 1927 Folin and Ciocalteu [22] made a critical study of the Folin and Looney methods for tyrosine and tryptophane and made certain changes in the technic which are claimed to make them entirely trustworthy. During this investigation a new colorimetric method for tyrosine was developed based on the Millon's test. While tyrosine ordinarily reacts very slowly with Millon's reagent in the cold, it was found that after tyrosine had been boiled with mercuric sulfate it reacted immediately and in the cold with this reagent. It was also found that sodium nitrite originally used by Nasse was just as good, if not better, than Millon's reagent. The new method for tyrosine consisted in the removal of tryptophane with mercuric sulfate, similar to the Folin and Looney procedure, then a comparison of colors developed by the unknown and a standard tyrosine solution when treated with sodium nitrite in the proper sulfuric acid concentration.

Almost simultaneously with the publication of Folin and Ciocalteu, Hanke [23] published a comparison of a method recently proposed by him with that of Folin and Looney and showed that in casein and egg

[20] Folin, O., and Looney, J. M., *J. Biol. Chem.*, 1922, li, 421.
[21] Looney, J. M., *J. Biol. Chem.*, 1926, lxix, 519.
[22] Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, 1927, lxxiii, 627.
[23] Hanke, M. T., *Proc. Am. Soc. Biol. Chem.*, 1927, xxi, 10.

albumin there is at least one factor besides tyrosine which reacts with the Folin and Looney phenol reagent.*

Determination of Tyrosine by Bromination

J. H. Millar [24] in 1903 showed that tyrosine could be brominated, forming a dibromo derivative, and that this reaction could be used for the estimation of this substance in simple mixtures of amino acids. A few years later Brown and E. T. Millar,[25] using the bromine reaction of J. H. Millar, showed that tyrosine was completely liberated at a very early stage in the hydrolysis by trypsin and that the bromine addition method for the estimation of tyrosine could be used in the presence of protein material if proper checks were employed. The solution containing tyrosine in hydrochloric acid was treated with potassium bromide and then titrated with 0.2 *N* sodium bromate. The liberated bromine was readily absorbed by the tyrosine. The end-point was marked with potassium iodide and starch as indicator, since the yellow bromine color at the end was not always distinct. From their data the tyrosine content of edestin is 4.06 per cent, a value considerably higher than that obtained by direct isolation (2.1%). The tyrosine content of no other proteins was reported. Auld and Mosscrop [26] used 0.05 *N* sodium bromate, with methyl violet or gentian violet as an outside indicator. They confirmed Brown and Millar's contention that tyrosine was liberated in the early stages of tryptic digestion and that the method was applicable to the estimation of tyrosine in the presence of proteins and other early cleavage products.

Plimmer and Eaves [27] studied the reaction more closely and found that, besides tyrosine, histidine, tryptophane and the products resulting from boiling tryptophane with strong acids, all absorb bromine. They outlined a procedure in which these interfering substances were not present in the tyrosine solution to be tested. The presence of proteins and of histidine was avoided by a preliminary precipitation with phosphotungstic acid. The presence of free tryptophane or its decomposition products was largely avoided by carrying out the hydrolysis with trypsin and stopping the digestion after six hours, at the end of which time it was thought that practically all the tyrosine and but little tryptophane had been split off. The procedure, as described by Plimmer,[3, p. 22] is briefly as follows: The protein was digested in faintly alkaline solution

* See footnote on page 149.

[24] Millar, J. H., Trans. Guinness Research Laboratory, 1903, i, pt. 1, 40; cited through Plimmer (3), p. 22.

[25] Brown, A. J., and Millar, E. T., *J. Chem. Soc.*, 1906, lxxxix, 145.

[26] Auld, S. J. M., and Mosscrop, T. D., *J. Chem. Soc.*, 1913, ciii, 281.

[27] Plimmer, R. H. A., and Eaves, E. C., *Biochem. J.*, 1913, vii, 297.

with trypsin for six hours, after which the solution was treated with 5 per cent phosphotungstic acid in 5 per cent sulfuric acid. An aliquot of the filtrate was then treated with 0.2 *N* sodium bromate solution followed by a 20 per cent sodium bromide solution. After half an hour the excess bromine was titrated with thiosulfate, using starch and sodium iodide as indicator. A correction for the bromine absorbed by the same volume of trypsin solution treated in the same manner was made.

Plimmer and Eaves determined the tyrosine content of a number of proteins by this method. Bromination after six hours' digestion gave values comparable with the gravimetric values, while bromination after 24 and 48 hours gave values comparable with those obtained by Folin and Denis. The authors concluded, therefore, that the bromination method, as they used it, was of little value for the determination of tyrosine in proteins. Although Fürth and Fleischmann [17] used the bromination method of Plimmer and Eaves, Fürth [18] and Fürth and Fischer [19] later criticized the method because bromine was reduced or absorbed by many organic compounds.

Plimmer and Phillips [28] in 1924 reported a further study on the bromination of the amino acids and described a procedure for the quantitative determination of tyrosine and histidine by bromination. Among the products of acid hydrolysis of a protein absorbing bromine were tyrosine, tryptophane and its decomposition products, histidine and cystine. During acid hydrolysis tryptophane is largely destroyed and it was found that the decomposition products were removed to a large extent in the humin. Histidine and tyrosine were separated by phosphotungstic acid. Cystine was so changed during hydrolysis that little remained in the phosphotungstic acid precipitate. Thus, except in the case of the keratins or other proteins containing a large amount of cystine, when it causes too high values for tyrosine, the procedure is claimed to give fairly accurate values for both tyrosine and histidine.

Determinations of Tyrosine Based on Millon's Test

Millon's reaction, which consists in the development of a red color when tyrosine is heated in contact with a mixture of mercuric nitrite and nitrate, is one of the most specific reactions for tyrosine. Weiss [29] in 1919 described a colorimetric method for the quantitative determination of tyrosine in protein hydrolysates. He simply diluted the

[28] Plimmer, R. H. A., and Phillips, H., *Biochem. J.*, 1924, xviii, 312.
[29] Weiss, M., *Biochem. Z.*, 1919, xcvii, 170.

unknown until it produced the same color as did a 1 : 50,000 tyrosine standard when both were treated with Millon's reagent.

In 1921 Thomas [14] also described a colorimetric method using Millon's reagent. The protein was hydrolyzed with sulfuric acid, filtered from humin, neutralized with barium hydroxide, acidified with nitric acid and filtered. Tryptophane was precipitated with mercuric nitrate and an aliquot of the filtrate, after decolorization, was treated with Millon's reagent and the color compared with a similarly treated tyrosine standard.

TABLE 6. *The Tyrosine Content of Casein as Determined by Different Methods.*

Investigator and Reference Number	Year	Tyrosine *Per Cent*	Method
Fischer [a]	1901	4.5	Isolated and weighed
Foline and Denis (7)	1912	6.5	Colorimetric (phenol reagent)
Haas and Trautmann (30)	1922	6.0	Colorimetric (phenol reagent)
Folin and Looney (20)	1922	5.3–5.4	Colorimetric (phenol reagent)
Weiss (29)	1919	5.6	Colorimetric (Millon's reaction)
Thomas (14)	1921	8.5[b]	Colorimetric (Millon's reaction)
Fürth and Fleischmann (17)	1922	3.5–4.5	Colorimetric (Millon's reaction)
Fürth and Fischer (19)	1924	5.9–8.0	Colorimetric (Millon's reaction)
Folin and Ciocalteu (22)	1927	6.5	Colorimetric (Millon's reaction)
Zuwerkalow (31)	1927	6.8–7.2	Colorimetric (Millon's reaction)
Fürth and Fleischmann (17)	1922	4.7–5.3	Bromine addition
Fürth (18)	1924	5.3–8.0	Bromine addition
Fürth (18)	1924	5.2	Colorimetric (diazo reaction)
Hanke (32)	1925	4.5	Colorimetric (diazo reaction)

[a] Fischer, 9., *Z. physiol. Chem.*, 1901, xxxiii, 151.
[b] Phenol index.

Haas and Trautmann,[30] using a method similar to the Millon procedure described by Weiss, reported the values for tyrosine in several proteins. Fürth,[18] however, after an attempt to use the Millon's reaction as a basis for tyrosine determinations, concluded that it was an unreliable means for the estimation of the tyrosine content of proteins. Nevertheless, later in the same year Fürth and Fischer,[19] after a comparison of several gravimetric and colorimetric procedures, concluded that a method involving Millon's reagent yielded the best results. In their procedure the protein was hydrolyzed with sulfuric acid and the hydrolysate treated with phosphotungstic acid. To the filtrate from the phosphotungstic acid precipitate, 5 per cent quinine sulfate was added in excess, the solution acidified and filtered. The colors developed by treating an aliquot of the filtrate and a suitable tyrosine standard with Millon's reagent and allowing to stand at room temperature for three-quarters of an hour, are compared in a colorimeter.

The determination of the tyrosine content of proteins by means of

[30] Haas, G., and Trautmann, M., *Z. physiol. Chem.*, 1923, cxxvii, 52.

Millon's reagent has recently been made by Zuwerkalow,[31] who claims to have determined the tyrosine content of proteins directly by the aid of glacial acetic acid without previous hydrolysis.

Hanke [32] criticized the Fürth and Fischer procedure as not being reliable "because the procedure is sensitive to slight and uncontrollable variations in the composition of the final reaction liquid" and Folin and Ciocalteu,[22] who also have described a quantitative method for tyrosine based on the Millon procedure (see p. 145), claimed that the different methods based on the Millon reaction heretofore proposed were not dependable, since the colored compound obtained was comparatively unstable, "yet had always been obtained by the aid of heat." The last statement is incorrect since Fürth and Fischer developed their color at room temperature. However, the different methods based on the Millon reaction have not given consistent results as is shown in Table 6.*

The Determination of Tyrosine by the Diazo Reaction

Hanke and Koessler [33] in 1922 published a colorimetric method for the quantitative estimation of phenols including tyrosine. The method is based upon the production of a yellow to red color when phenols are treated in alkaline (Na_2CO_3) solution with diazotized sulfanilic acid. Tyrosine and tyramine differ from all other phenols in that the color developed fades rapidly and is not proportional to the amount of tyrosine or tyramine introduced. If at the same time, however, the liquid is treated with a solution of sodium hydroxide and then with a small amount of hydroxylamine hydrochloride, a violet color, which is directly proportional to the amount of tyrosine or tyramine present, develops and remains unchanged for at least fifteen minutes.

This method was not applicable to the estimation of tyrosine in protein and protein hydrolysates because some other amino acids produced a color similar to that given by tyrosine. Hanke [34, 32] in 1925 suggested a procedure which was claimed to be applicable to the esti-

[31] Zuwerkalow, D., *Z. physiol. Chem.*, 1927, clxiii, 185.
[32] Hanke, M. T., *J. Biol. Chem.*, 1925, lxvi, 489.
* In a recent paper (*J. Biol. Chem.*, 1928, lxxix, 587), Hanke reported the results of a critical study of the methods for the estimation of tyrosine by the colorimetric phenol-reagent method of Folin and Looney, the Millon's reaction of Folin and Ciocalteu, as well as his own colorimetric method using alkaline diazotized sulfanilic acid. He answered much of the criticism which had been directed against his method and concluded in part: that the tyrosine figures reported by him in 1925 were from 0.3 to 0.6 per cent low, due, not to the interference of tryptophane (as suggested by Looney), but to the fact that a small amount of tyrosine was carried down with the silver precipitate of histidine; that the phenol-reagent method of Folin and Looney is not reliable except for pure tyrosine solutions; that the modified Millon procedure of Folin and Ciocalteu gives uniform results, which are identical with those obtained by the method of Hanke and Koessler. He suggests that the tyrosine content of a protein can be accurately determined by conducting a colorimeter Millon determination on the precipitate resulting from the precipitation of tyrosine by mercuric acetate in the presence of sodium chloride.
[33] Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1922, l, 235, 271.
[34] Hanke, M. T., *J. Biol. Chem.*, 1925, lxvi, 475.

mation of tyrosine in protein hydrolysates. The protein was hydrolyzed with sulfuric acid. Histidine was then precipitated as the silver complex and its amount estimated colorimetrically by the method of Koessler and Hanke.[35] The filtrate from the histidine silver was then boiled with mercuric acetate and treated with sodium chloride, which precipitates the tyrosine as a mercury compound. This tyrosine precipitate was dissolved in hydrochloric acid, freed from mercury with hydrogen sulfide, and the tyrosine estimated in the filtrate from the mercuric sulfide precipitate by the method of Hanke and Koessler,[33] described above. Hanke [23] has recently published a brief comparison of his method with the Folin and Looney method and attempted to show that the invariably higher results obtained in the latter method were erroneous. The tyrosine values obtained by Hanke's method are more nearly like those obtained by the gravimetric methods.

Fürth and Fleischmann,[17] using a diazo method for tyrosine similar to that used by Weiss and Ssobalew [36] for histidine, reported values for tyrosine much like those reported by the other colorimetric methods. Fürth [18] in 1924, however, from a study of several methods for the determination of tyrosine, concluded that none of the methods to date, based upon the diazo reaction, bromine addition, the phosphotungstic-phosphomolybdic reagent, Millon's reaction, and certainly not the gravimetric methods, provide a reliable means for the estimation of the tyrosine content of proteins.

The tyrosine content of casein as reported by investigators using one or more of the previously described methods are tabulated in Table 6. From an examination of this table as well as of the publications mentioned above it is extremely doubtful that any method yet proposed for the estimation of the tyrosine content of proteins is entirely satisfactory.

CYSTINE

Gravimetric Determinations of Cystine

Cystine, like tyrosine, is almost insoluble in neutral aqueous solutions. They differ in this respect from almost all of the other amino acids, and use has been made of this fact in the isolation and determination of these two amino acids in protein hydrolysates. Cystine and tyrosine, however, resemble each other so closely in their solubility in water, in alkali, and in acid solutions, that their separation is very difficult.

[35] Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 497, 521.
[36] Weiss, M., and Ssobalew, N., *Biochem. Z.*, 1913, lviii, 119.

Mörner [37] and Embden [38] in 1899 were the first to isolate cystine from the products of acid hydrolysis of proteins. Mörner hydrolyzed hair, horn, etc., by heating the protein with five times its quantity of 13 per cent hydrochloric acid on a water-bath under a reflux condenser for six to seven days. The solution was decolorized with charcoal, concentrated *in vacuo,* and the residue taken up in 60 to 70 per cent alcohol. On neutralizing with soda a mixture of cystine and tyrosine separated out. The two substances were separated by fractional crystallization from ammonia.

Embden hydrolyzed the protein (horn) with hydrochloric acid, neutralized the hydrolysate, and allowed it to stand for not more than 24 hours. After filtering off the humin, the filtrate was acidified, decolorized, and allowed to stand in a cool place. The mixture of tyrosine and cystine which crystallized out was separated by dissolving out the tyrosine in very dilute nitric acid, the solution of tyrosine being followed by microscopic observations. Friedmann [39] also made use of the fact that cystine is the more insoluble in dilute mineral acids, while Abderhalden [40] separated cystine and tyrosine by dissolving in ammonia and almost neutralizing with acetic acid; tyrosine was precipitated. On strongly acidifying with glacial acetic acid the cystine precipitated. For the purpose of obtaining large amounts of cystine, Folin [41] described a method for the separation of cystine and tyrosine. The hydrochloric acid hydrolysate was neutralized to Congo red with sodium acetate, when cystine crystallized out. On diluting the filtrate and allowing it to stand the tyrosine slowly separated out. A similar method was proposed by Denis,[42] who used a hot solution of sodium acetate for the purpose of neutralization.

Winterstein [43] in 1901 showed that cystine was precipitated by phosphotungstic acid, but this property seems to have been little used. Van Slyke [44] makes use of the precipitation of cystine with phosphotungstic acid in his nitrogen distribution method, and Plimmer [5] showed that the separation of cystine and tyrosine was quantitative when the former was precipitated with phosphotungstic acid, but there was some loss in the recovery of cystine from the precipitate.

Hopkins and Cole [45] used a solution of mercuric sulfate in 5 per cent

[37] Mörner, K. A. J., *Z. physiol. Chem.,* 1899, xxviii, 595; 1901-02, xxxiv, 207.
[38] Embden, G., *Z. physiol. Chem.,* 1900, xxxii, 94.
[39] Friedmann, E., *Beitr. chem. Physiol. Path.,* 1902 iii, 1.
[40] Abderhalden, E., *Z. physiol. Chem.,* 1903, xxxvii, 484.
[41] Folin, O., *J. Biol. Chem.,* 1910, viii, 9.
[42] Denis, W. *J. Biol. Chem.,* 1911, ix, 365.
[43] Winterstein, E., *Z. physiol. Chem.,* 1901, xxxiv, 153.
[44] Van Slyke, D. D., *J. Biol. Chem.,* 1911, x, 15.
[45] Hopkins, F. G., and Cole, S. W., *J. Physiol.,* 1901-02, xxvii, 418.

sulfuric acid to precipitate the cystine, the mercury compound of tyrosine being soluble in the 5 per cent sulfuric acid. This method of separation has been used by Patten,[46] Osborne and Clapp,[8] Hopkins and Savory [47] and others. Plimmer [5] made a study of this means of separation and concluded that the cystine was not completely precipitated under these conditions and that the tyrosine recovered was impure.

Plimmer [5] stated that tyrosine and cystine may be quantitatively separated by means of absolute alcohol saturated with hydrogen chloride. The tyrosine is readily converted into its ethyl ester hydrochloride while cystine is not. The tyrosine ester was dissolved in warm alcoholic hydrochloric acid and any dissolved cystine was precipitated with absolute alcohol. The undissolved cystine was filtered off, washed free of acid with absolute alcohol, and recrystallized from ammonia.

Harris [48] also pointed out the difficulty of complete separation of cystine and tyrosine by methods based on the slight difference in solubilities of the two in acid and alkaline solutions. He pointed out that the isoelectric points of the two were not far apart, that of cystine being pH 4 and that of tyrosine 5.4, and that cystine was not completely thrown out of its concentrated solutions even at its isoelectric point. He showed that the presence of metallic salts and organic matter interfered with the separation of cystine and, therefore, appreciably more cystine could be recovered by neutralization with soda as in Abderhalden's method than when the solution was buffered by the addition of necessarily larger quantities of sodium acetate as in Folin's method. A new method for the estimation of cystine in hydrolysis mixtures was proposed and used by Harris. The method depends upon the precipitation of cystine with the mercuric sulfate reagent, decomposition of the mercury precipitate with hydrogen sulfide, and precipitation of the resulting cystine with copper hydroxide. According to Harris, the tyrosine, which is also precipitated by mercuric sulfate, forms a mercury derivative soluble in 5 per cent sulfuric acid solution, and it yields a more soluble copper salt, while tryptophane is destroyed during acid hydrolysis. The yield of cystine, which was isolated in a highly purified form and weighed, was in excess of that hitherto obtained in the proteins with which he was working. Harris objected to the methods of Okuda and of Folin and Looney, to be described below, because of the possibility of loss during decolorization and also because the presence of other amino acids gave rise to complications.

[46] Patten, A. J., *Z. physiol. Chem.*, 1903, xxxix, 350.
[47] Hopkins, F. G., and Savory, H., *J. Physiol.*, 1911, xlii, 189.
[48] Harris, L. J., *Proc. Royal Soc. (London)*, 1922-3, xciv, B, 441.

The Titration Method of Okuda

In 1919 Okuda [49] described a method for the determination of cystine by titration with bromate solution, but this method could not be used in the presence of tyrosine and tryptophane; histidine was said not to interfere. Later [50] Okuda described, in a series of papers, a method which was applicable in the presence of tyrosine, tryptophane and all other protein cleavage products. In this method cystine is reduced with zinc and hydrochloric acid and the resulting cysteine titrated in hydrochloric acid solution in the presence of I^- ions with a standard iodate solution.

The Colorimetric Method of Folin and Looney

At the same time they described their methods for the determination of tyrosine and of tryptophane, Folin and Looney [20] also proposed a colorimetric method for the quantitative estimation of cystine. The method is based on the production of a blue color when cystine is treated in the presence of sodium sulfite with phosphotungstic acid. The color produced was matched against the color similarly developed from a standard cystine solution. It was stated that the reaction was specific for cystine among the protein hydrolytic products. A slight modification of this procedure has been described by Hunter and Eagles.[51]

The Naphthoquinone Method of Sullivan

In 1925 Sullivan [52] described a qualitative test for cystine which depended upon the production of a red color when cysteine, produced by the reduction of cystine with sodium cyanide, was treated with sodium-β-naphthoquinone-4-sulfonate in the presence of alkali and a reducing agent such as sodium sulfite. In 1927 Sullivan [53] modified this procedure to make it applicable to the quantitative estimation of cystine in protein hydrolysates. The color developed by a described procedure was compared with a color similarly developed from a standard cystine solution.

[49] Okuda, Y., *J. Col. Agr. Imp. Univ. Tokyo,* 1919, vii, 69; cited through *Exp. Sta. Rec.,* 1920 xliii, 505.
[50] Okuda, Y., *J. Sci. Agr. Soc. (Japan)*, 1923, ccliii, i, cited through *Chem. Abstr.,* 1924, xviii, 2014, 3613; *J. Chem. Soc. (Japan)*, 1924, xlv, 18, cited through *Chem. Abstr.,* 1924, xviii, 3400; *J. Biochem. (Japan)*, 1925, v, 201, cited through *Chem. Abstr.,* 1926, xx, 1094.
[51] Hunter, G., and Eagles, B. A., *J. Biol. Chem.,* 1927, lxxii, 177.
[52] Sullivan, M. X., *Abst. Bact.,* 1925, ix, 37.
[53] Sullivan, M. X., *Proc. Am. Soc. Biol. Chem.,* 1927, xxi, 14.

A Biological Method

Sherman and Woods [54] have described a method for the quantitative determination of cystine by means of feeding experiments with rats. On the assumption that cystine is the first limiting amino acid of the proteins of milk, the growth resulting from the addition to the basal diet, in which whole-milk powder furnished practically all of the protein, of definite amounts of the cystine containing material, is used as a quantitative measure of the cystine content of the material. It would seem that a careful control of the food intake of the rats would be essential before the results obtained can be more than roughly comparative in their significance.

Van Slyke's Method

As described in more detail in Chapter II, Van Slyke [44] calculated the cystine content of proteins from the sulfur content of the phosphotungstic acid precipitate from an acid hydrolysis. The accuracy of the cystine values by this method is seriously vitiated by the solubility of the cystine phosphotungstate and also to a greater extent by the partial decomposition of cystine into products not precipitated by phosphotungstic acid during the acid hydrolysis. The partial destruction of cystine by mineral acids during hydrolysis by these agents is a source of error in all methods involving acid hydrolysis of the proteins. Alkaline hydrolysis cannot be used for cystine for it is even more readily destroyed by boiling alkalies.

A critical study of the errors involved in the Van Slyke method for the estimation of cystine has been made by Plimmer and Lowndes,[55] who also describe a modified Van Slyke procedure in which the cystine is determined in the phosphotungstic acid precipitate without the removal of the phosphotungstic acid.

While it has been generally regarded that the cystine values found by the Van Slyke method are too low, it is of interest to note the close agreement existing in so many cases between the Van Slyke and the Folin and Looney methods as found by Jones, Gersdorff and Moeller.[56]

The Determination of Cystine in Urine

The determination of cystine in the urine in cases of cystinuria has led to the development of methods somewhat different than those used in the determination of cystine in protein hydrolysates. Mester [57] intro-

[54] Sherman, H. C., and Woods, E., *J. Biol. Chem.*, 1925, lxvi, 29.
[55] Plimmer R. H. A., and Lowndes, J., *Biochem. J.*, 1927, xxi, 247.
[56] Jones, D. B., Gersdorff, C. E. F., and Moeller, O., *J. Biol. Chem.*, 1924, lxii, 183.
[57] Mester, B., *Z. physiol. Chem.*, 1890, xiv, 109.

duced an indirect method for cystine in cystinuric urine based on the determination of organic or neutral sulfur obtained by subtracting from the total sulfur of the urine the total sulfate sulfur. This organic or neutral sulfur was assumed to be derived entirely from cystine.

The method which has been used most widely for the determination of cystine in cystinuric urine is the direct isolation method of Gaskell.[58] The method, which has been modified in minor detail by a number of investigators, consists in the removal of phosphates by calcium chloride and ammonia, and acidification of the filtrate with acetic acid. The insolubility of cystine in acetic acid is increased by the addition of an equal volume of alcohol or acetone. The precipitated cystine is filtered, reprecipitated from acetic acid and alcohol or acetone, filtered, dried, and weighed. Magnus-Levy [59] has recently modified the method somewhat. He preferred the use of alcohol over acetone and, after the first precipitation of cystine by alcohol, the cystine was filtered off and its purity determined by polariscopic methods.

The Folin and Looney colorimetric method for the determination of cystine in acid hydrolysates of proteins, based upon the reduction of cystine to cysteine by sodium sulfite and the subsequent development of a blue color when the cysteine is treated with Folin's uric acid reagent in the presence of sodium carbonate, was made applicable to the determination of cystine in cystinuric urines by Looney.[60] Lewis and Wilson [61] have made a critical study of the latter method as well as of the gravimetric method of Gaskell and the alcohol precipitation-polariscopic method of Magnus-Levy. They showed that the methods involving precipitation of the cystine may be as much as 40 to 50 per cent too low if the amounts of cystine are of the order of magnitude usually present in cystinuric urine. The Looney colorimetric method was found to give the most satisfactory results.

TRYTOPHANE

Tryptophane was shown to be a constituent of protein first by Hopkins and Cole,[45] who in 1901 isolated 1.5 per cent from casein. It is now well known that this amino acid is indispensable for the normal nutrition of animals and it is therefore important to have as much information as possible concerning its quantitative distribution among the various food proteins. With this incentive a large number of methods have been proposed for the quantitative estimation of trypo-

[58] Gaskell, J. F., *J. Physiol.*, 1907-08, xxxvi, 142.
[59] Magnus-Levy, A., *Biochem. Z.*, 1925, clvi, 150.
[60] Looney, J. M., *J. Biol. Chem.*, 1922, liv, 171.
[61] Lewis, H. B., and Wilson, R. H., *J. Biol. Chem.*, 1926, lxix, 125.

phane in proteins. Nevertheless until quite recently reliable data concerning the tryptophane content of proteins were few.

As has been previously discussed, tryptophane is rapidly destroyed by boiling with mineral acids, so it is not obtained from acid hydrolysis of proteins but is prepared from the hydrolysates resulting from tryptic digestion in alkaline solution or from alkaline hydrolysis of the protein. In fact, the decomposition of tryptophane by boiling with acids has been made use of in an indirect method for estimating this amino acid. Gortner and his associates have shown that the "humin" formed during the acid hydrolysis of proteins in the presence of aldehydes was due to tryptophane, and Holm and Greenbank [62] conclude, from a consideration of the work of Gortner and Holm,[63] that, "In the presence of a definite *optimum* amount of a certain aldehyde, the amount of 'humin' formed as measured by the nitrogen content, is a fairly accurate estimate of the amount of tryptophan." From the data given by Gortner and Holm, Holm and Greenbank calculate that the "humin" formation method of these authors indicates the presence of 5.05 per cent of tryptophane in fibrin. This value agrees closely with the value of 5.00 per cent obtained by Holm and Greenbank with their colorimetric method (see below). The "humin" formation method has not been established, however, as a general method of tryptophane analysis and has not been used by investigators other than the originators. All other tryptophane methods may be conveniently discussed under the following headings: (*a*) gravimetric methods, (*b*) the glyoxylic acid colorimetric method, (*c*) a method based on the estimation of indole produced by the action of fecal bacteria, (*d*) the *p*-dimethyl-aminobenzaldehyde colorimetric method, (*e*) bromine titration method, (*f*) Voisenet's formaldehyde method, (*g*) the colorimetric estimation by means of Folin and Denis' phenol reagent, and (*h*) Onslow's differential nitrogen method.

Gravimetric Methods

By the method of Hopkins and Cole,[45] who first isolated tryptophane, the protein was digested by trypsin in alkaline solution until a maximum coloration with bromine water was obtained. After making the solution to contain 5 per cent sulfuric acid, the mercuric sulfate reagent (10% mercuric sulfate in 5% sulfuric acid) was added as long as a precipitate, containing tryptophane, tyrosine, and cystine, formed. The precipitate was freed from tyrosine by washing with 5 per cent

[62] Holm, G. E., and Greenbank, G. R., *J. Am. Chem. Soc.*, 1923, xlv, 1788.
[63] Gortner, R. A., and Holm, G. E., *J. Am. Chem. Soc.*, 1917, xxxix, 2477.

sulfuric acid, in which the tyrosine compound is soluble. The precipitate was then decomposed with hydrogen sulfide and the solution containing tryptophane and cystine after the removal of hydrogen sulfide was again made to contain 5 per cent sulfuric acid and fractionally precipitated with the mercuric sulfate reagent. Cystine was precipitated first. This procedure was repeated. The tryptophane precipitate was finally decomposed with hydrogen sulfide, the mercury sulfide filtered off and the filtrate freed from hydrogen sulfide, then from sulfuric acid by barium, and concentrated, when tryptophane crystallized out. The tryptophane was recrystallized from water and the yield represented its amount in the protein. By this procedure Hopkins and Cole isolated 1.5 per cent of tryptophane from casein. This amount is considerably larger than that found by several colorimetric procedures.

Neuberg and Popowsky [64] and Abderhalden and Kempe [65] have introduced some alterations in the procedure. The former evaporated to one quarter volume, allowing considerable tyrosine to crystallize out, and this was filtered off before precipitation with mercuric sulfate. The latter investigators evaporated *in vacuo* for the same purpose. The former decomposed the mercuric sulfate precipitate by hydrogen sulfide in a warm alkaline solution and removed the sulfuric acid in the last operation with lead carbonate in ammoniacal solution, the lead being subsequently removed with hydrogen sulfide. Abderhalden and Kempe suggested the removal of hydrogen sulfide by means of a current of carbon dioxide. These investigators, however, isolated less tryptophane from casein than did Hopkins and Cole.

Dakin [66] combined his butyl alcohol extraction method with the Hopkins and Cole procedure for the estimation of tryptophane in proteins as follows: Casein was digested in 1 per cent sodium carbonate solution for seven days with an active pancreas extract, and the solution was precipitated once as described by Hopkins and Cole. The precipitate was decomposed with hydrogen sulfide and, after the exact removal of the sulfuric acid with barium hydroxide, the solution was concentrated under diminished pressure to a small volume. This concentrated solution was then extracted with butyl alcohol in a small continuous extractor. In a short time tryptophane began to settle out and at the end of twelve hours the extraction was complete. Most of the tryptophane was crystallized from the butyl alcohol, but a small quantity was obtained from the mother liquors by crystallization from absolute alcohol. In this

[64] Neuberg, C., and Popowsky, N., *Biochem. Z.*, 1907, ii, 357.
[65] Abderhalden, E., and Kempe, M., *Z. physiol. Chem.*, 1907, lii, 207.
[66] Dakin, H. D., *Biochem. J.*, 1918, xii, 290.

manner Dakin obtained 1.7 per cent of tryptophane from casein on the first attempt.

Osborne and Harris [67] as early as 1904, using a method similar to that of Hopkins and Cole, roughly determined the tryptophane content of a number of vegetable proteins.

For the direct isolation of tryptophane from proteins tryptic digestion has ordinarily been used. Waterman [68] has attempted the Hopkins and Cole isolation procedure with baryta hydrolysates, but concluded that this means of hydrolysis could not be carried to completion without the destruction of some of the tryptophane. This method of hydrolysis, however, was claimed to have the advantages of being quicker and also of destroying the cystine, the absence of which is of considerable advantage in the recrystallization of tryptophane.

The Glyoxylic Acid Colorimetric Method

Proteins containing tryptophane give a reddish-violet to blue color when treated with glyoxylic acid and sulfuric acid. This test is known as the Adamkiewicz-Hopkins test and is sensitive to a tryptophane concentration of 1 : 100,000. This reaction has been made the basis of a colorimetric method for the determination of tryptophane in proteins by Fasal.[69] About 0.1 gram of the protein is dissolved in a mixture of 6 cc. of concentrated sulfuric acid and 2 cc. of glyoxylic acid. The maximum color develops in about one hour. A series of controls containing tryptophane in proportions of 1 : 1000 to 1 : 50,000 or more are made up, and the control corresponding most closely to the unknown is compared with it in a Duboscq colorimeter.

In most cases the colors could be matched fairly well, but in some cases the color comparison was difficult due to brown coloration of some proteins. In these cases Fasal [70] found it possible to make the comparison if color filters were used. By this method Fasal [69] found 0.65 per cent tryptophane in casein. This value is considerably below that actually isolated by Hopkins and Cole. Fasal [70] also used the method in a study of the tryptophane content of normal tissue and of malignant tumors.

Method Based upon the Estimation of Indole Produced by Fecal Bacteria

Tryptophane is decomposed by putrefactive bacteria with the formation of indole. Sanders and May [71] have proposed a method for the

[67] Osborne, T. B., and Harris, I. F., *Z. anal. Chem.*, 1904, xliii, 376.
[68] Waterman, H. C., *J. Biol. Chem.*, 1923, lvi, 75.
[69] Fasal, H., *Biochem. Z.*, 1912, xliv, 392.
[70] Fasal. H., *Biochem. Z.*, 1913, lv, 88.
[71] Sanders, J. A., and May, C. E., *Biochem. Bull.*, 1912-13, ii, 373.

estimation of tryptophane based on the production of indole by fecal bacteria. The pancreatic digest of the protein was inoculated and incubated with fecal bacteria. The indole was distilled and treated with nitrous acid. The color produced was compared with the color similarly produced from a standard indole solution. The method assumes that the tryptophane is completely converted into indole, although such is not necessarily the case. Sanders and May reported 1.6 per cent of tryptophane in casein; this is 0.1 per cent higher than that isolated by Hopkins and Cole. Moraczewski,[72] however, using a similar method, obtained only 0.6 per cent tryptophane in casein.

The *p*-Dimethylaminobenzaldehyde Colorimetric Method

Rhode [73] was the first to observe that tryptophane gave a blue color with *p*-dimethylaminobenzaldehyde, but Herzfeld [74] was the first to determine quantitatively the tryptophane content of proteins by the colorimetric and spectrophotometric comparison of the colors produced by the tryptophane in protein with *p*-dimethylaminobenzaldehyde and concentrated hydrochloric acid. A blue color is given by this reagent with 1 part of tryptophane in 1,000,000. In this procedure the protein was digested at 37° C. in a 0.5 per cent sodium carbonate solution with pancreatin in the presence of chloroform and xylene. An aliquot of this digest was treated with *p*-dimethylaminobenzaldehyde and concentrated hydrochloric acid. After standing thirty minutes the color was compared with the color of a solution of ammoniacal copper sulfate previously standardized in terms of a solution of pure tryptophane similarly treated with *p*-dimethylaminobenzaldehyde.

The tryptophane content of a large number of proteins was determined, but compared with most other determinations, the values are low. These low values may have been due to the short digestion period (twenty-four hours), for Hopkins and Cole found that the maximum coloration given by a tryptic digest was not reached until after several days; or they may have been due, as explained by Holm and Greenbank,[62] to the fact that the reaction mixtures of either standards or unknowns were not allowed to react long enough to produce the most intense color.

Herzfeld reported but 0.51 per cent tryptophane in casein. This is less than one-third that actually isolated by Dakin and exactly one-third that isolated by Hopkins and Cole. This value for casein as well as all other values by Herzfeld are therefore probably much too low.

[72] Moraczewski, W., *Biochem. Z.*, 1913, li, 340.
[73] Rhode, E., *Z. physiol. Chem.*, 1905, xliv, 161.
[74] Herzfeld, E., *Biochem. Z.*, 1913, lvi, 258.

Using Herzfeld's *p*-dimethylaminobenzaldehyde colorimetric reaction, Kurchin [75] determined the tryptophane content of normal and pathological kidneys; normal kidneys contained slightly more tryptophane than pathological.

Thomas [76] used Herzfeld's method but digested his proteins completely and obtained considerably higher values than did Herzfeld. He reported 1.7 to 1.8 per cent tryptophane in casein. This value agrees closely with the amount isolated by Dakin and by Hopkins and Cole. According to Thomas the chief precaution to take in Herzfeld's method is to insure complete solution of the protein used.

May and Rose [77] proposed a slightly different procedure involving the use of the Ehrlich reagent (*p*-dimethylaminobenzaldehyde). They hydrolyzed the protein with hydrochloric acid in the presence of the aldehyde. In this manner the greenish or reddish shades often produced by tryptic digestion or alkaline hydrolysis are obviated and only an intense blue proportional to the tryptophane present and permanent for at least ten days is obtained. Also because the shade of blue developed by the reaction of *p*-dimethylaminobenzaldehyde with tryptophane in the presence of other protein cleavage products differs somewhat from that obtained when the reaction takes place with tryptophane alone, May and Rose used casein as a standard. In their calculations the tryptophane content of casein was assumed to be 1.5 per cent. From the fact that Dakin actually isolated 1.7 per cent and more recent investigations have indicated a still larger value, it is probable that the values of May and Rose are correspondingly too low.

The procedure of May and Rose is briefly as follows: A weighed portion of the protein (0.05 to 0.1 gram) is mixed with 50 cc. of concentrated hydrochloric acid, 50 cc. of water and 1 cc. of the reagent (5% *p*-dimethylaminobenzaldehyde in 10% sulfuric acid). The mixture is incubated at 35° C. for 24 hours and then allowed to stand 24 hours or longer at room temperature. The color produced is matched in a colorimeter against a standard produced similarly from 0.1 gram of casein hydrolyzed and diluted to 100 cc.

Holm and Greenbank [62] have examined the Herzfeld method and claim that while it is capable of giving accurate results, practically all of the previous users of this method have made the common error of not attaining the maximum color in either the standard or the unknown. These workers used a method similar to that used by May and Rose except a pure tryptophane standard was employed. They

[75] Kurchin, E., *Biochem. Z.*, 1914, lxv, 451.
[76] Thomas, P., *Ann. Inst. Pasteur*, 1920, xxxiv, 701.
[77] May, C. E., and Rose, E. R., *J. Biol. Chem.*, 1922, liv, 213.

apparently show that under the conditions used by May and Rose a digestion period of about eight days is necessary in order to obtain the maximum color.

The percentage tryptophane found in casein by these investigators was 2.24, one of the highest values so far reported. They claim that the Herzfeld method properly used may be used for the determination of tryptophane in solution and in proteins with and without previous digestion with enzymes. They recommend, however, that the proteins be first digested with enzymes.

Jones, Gersdorff and Moeller,[56] who determined the tryptophane and cystine content of a large number of proteins, chose the procedure of May and Rose as the one capable of giving the most reliable results. Adopting the suggestion of Holm and Greenbank, that a 24-hour digestion at 37° C. followed by an interval of 40 hours standing at room temperature was not sufficient to produce the maximum color, they digested for 24 hours at 37° C., and made color comparisons until the maximum color was produced. The time of digestion necessary to produce the maximum color varied with different proteins.

These investigators also used casein as their standard, but first carefully determined its tryptophane content by the same procedure, using for this purpose a standard consisting of zein (shown to contain no tryptophane) to which a known quantity of pure tryptophane had been added. The average of several closely agreeing determinations gave 2.20 as the percentage of tryptophane in casein. All subsequent determinations were made with a casein standard, which was assumed to contain 2.2 per cent of tryptophane.

Determination of Tryptophane by Bromine Titration

Levene and Rouiller [78] suggested that, since tryptophane was more or less readily decomposed by concentration of its aqueous solutions in the original Hopkins and Cole method, the tryptophane in the mercuric sulfate precipitate be titrated with bromine. The protein was digested by trypsin, the digest precipitated with mercuric sulfate, and the precipitate decomposed with hydrogen sulfide in much the same manner as described in the Hopkins and Cole procedure. An aliquot of this solution was then titrated with bromine water in the presence of amyl alcohol until the purple color of the amyl alcohol became yellow.

Tyrosine and cystine, both of which are precipitated along with tryptophane by the mercuric sulfate reagent, absorb bromine. Tyrosine may be eliminated from the precipitate either by avoiding an excess of

[78] Levene, P. A., and Rouiller, C. A., *J. Biol. Chem.*, 1906-07, ii, 481.

mercuric sulfate or by washing the precipitate with 5 per cent sulfuric acid in which the tyrosine compound is soluble. If any appreciable amount of cystine is present a correction may be applied from an estimation of the cystine content of the solution by means of a sulfur determination. No analyses by this method have been reported.

Estimation of the tryptophane content of proteins by bromination was also proposed by Homer,[79] who advocated the use of barium hydroxide as a hydrolyzing agent in tryptophane determinations. The protein was hydrolyzed by heating with barium hydroxide on a steam-bath for 40 hours. In the absence of metallic salts, tryptophane was found to be stable towards baryta. After hydrolysis barium was removed with sulfuric acid. Tryptophane was then precipitated with the mercuric sulfate reagent, the tyrosine washed out with 5 per cent sulfuric acid, and the mercury-tryptophane compound decomposed in the usual manner. The filtrate freed from hydrogen sulfide was treated with phosphotungstic acid in order to remove polypeptides which also absorb bromine. After removal of phosphotungstic acid with barium and the barium with sulfuric acid, the filtrate was made up to a definite volume and an aliquot treated with sodium bromate and bromide solutions from which bromine was liberated by acid. The excess of bromine was titrated with thiosulfate. Cystine does not interfere, since it is completely destroyed by the alkaline hydrolysis.

The tryptophane content of casein was determined in this way, the results obtained varying from 0.99 to 1.59 per cent. No determinations upon other proteins were made.

Voisenet's Formaldehyde Method

Voisenet's [80] qualitative test for tryptophane, which consists of a violet coloration of a solution containing tryptophane on the addition of hydrochloric acid in excess and a trace of formaldehyde in the presence of sodium or potassium nitrite, has been made the basis of methods for the quantitative estimation of tryptophane. Fürth and Nobel [81] described such a method and, comparing the color produced by the unknown with that similarly produced from a standard tryptophane solution, reported that tryptophane content of several proteins. They reported a tryptophane content of casein as about 2 per cent. This paper was followed immediately by one of Fürth and Lieben,[82] who made a more systematic investigation of the various factors affecting the determination of tryptophane by this method. Using a slightly im-

[79] Homer, A., *J. Biol. Chem.*, 1915, xxii, 369.
[80] Voisenet, E., *Bull. soc. chim.*, 1905, [3], xxxiii, 1198.
[81] Fürth, O., and Nobel, E., *Biochem. Z.*, 1920, cix, 103.
[82] Fürth, O., and Lieben, F., *Biochem. Z.*, 1920, cix, 124.

proved technic they determined the tryptophane content of a number of proteins as well as that of normal tissues and organs of the body. It is claimed that the method is applicable to the determination of tryptophane in proteins with or without previous hydrolysis. Errors amounting to 10 to 20 per cent in the method were admitted.

The investigation concerned with the determination of tryptophane was continued by Fürth and Dische,[83] who studied various standard methods for hydrolysis as they modified tryptophane estimations according to a variety of procedures. The use of the Voisenet reaction was maintained with some modifications of the procedure used by Fürth and Lieben. Alkaline hydrolysis in the cold may be used. The use of a protein with a known tryptophane content as a standard was recommended over the pure tryptophane standard. The protein used was Hammarsten's casein and the tryptophane content of this protein was determined to be 1.7 per cent. A comparison of the values for the tryptophane content of a number of proteins as obtained by different methods is given in the report of this work.

Fürth's method for the estimation of tryptophane based on the Voisenet reaction has received much adverse criticism. Kretz [84] used a modification of Fürth's method for the localization of tryptophane in plant cells. He indicated that Fürth's method was unreliable for quantitative work, because it was so sensitive to slight variations in the reagents or in the procedure. Lüscher [85] attempted to determine the tryptophane content of Bence-Jones' protein and criticized Fürth and Lieben's method on the basis that the color obtained with formaldehyde and pure tryptophane is blue and that with proteins containing tryptophane is more or less reddish. This criticism, however, cannot be made of Fürth and Dische's [83] modification. Lüscher claims that the Fürth and Lieben method gives values from 30 to 60 per cent too high. He proposes the use of benzaldehyde in the place of formaldehyde and gives several reasons for doing so.

Kiyotaki,[86] working in Fürth's laboratory, found an average of 3.6 per cent tryptophane in hemoglobin although Fürth and Lieben report none. He points out a significant cause for criticism in that the concentration of the protein greatly affects the results; weak solutions are likely to produce no color. Severe criticism of the Fürth methods has also come from Hunter and Borsook,[87] Folin and Looney,[20] and Looney.[21]

[83] Fürth, O., and Dische, Z., *Biochem. Z.*, 1924, cxlvi, 275.
[84] Kretz, F., *Biochem. Z.*, 1922, cxxx, 86.
[85] Lüscher, E., *Biochem. J.*, 1922, xvi, 556.
[86] Kiyotaki, U., *Biochem. Z.*, 1923, cxxxiv, 322.
[87] Hunter, A., and Borsook, H., *J. Biol. Chem.*, 1923, lvii, 507.

Komm and Böhringer [88] described a modification of the Fürth and Lieben procedure similar in some respects to that adopted by Kretz. He obtained tryptophane values for casein and for Witte peptone in close agreement with those reported by Fürth and Lieben.

Kraus [89] proposed the use of still another aldehyde in the detection of tryptophane, *i.e.*, vanillin hydrochloride, because of the greater color intensity produced. However, she was unable to make quantitative use of the method because of interfering colors produced by other products of the digestion.

Finally Tillmans and Alt [90] have proposed a method which differs from the Fürth and Lieben application of the Voisenet reaction chiefly in the use of sulfuric instead of hydrochloric acid with the formaldehyde. By means of this modification the tryptophane content of a large number of proteins was determined, that of casein being placed at 1.60 per cent. Fürth [91] and Tillmans and Alt [92] later became involved in some controversy over this method.

The Colorimetric Estimation of Tryptophane by Means of Folin and Denis' Phenol Reagent

In 1922 Folin and Looney [20] proposed colorimetric methods for the quantitative estimations of tyrosine, tryptophane and cystine. The tyrosine (page 145) and cystine (page 153) methods have been previously described. In their methods for tyrosine and for tryptophane, each depends upon the development of a blue color with the phosphomolybdic-phosphotungstic acid phenol reagent of Folin and Denis.[11] The separation of tyrosine and tryptophane was accomplished by means of mercuric sulfate and sulfuric acid in the usual manner. For the determination of tryptophane, Folin and Looney dissolve the mercuric sulfate, which contains the tryptophane, in an excess of sodium cyanide. The tryptophane in this solution is then determined by treating the solution with the phenol reagent under the proper conditions and comparing the color produced with that produced from a standard tryptophane solution similarly treated. In the method of Folin and Looney hydrolysis of the protein was effected by means of baryta. The tryptophane content of casein was found to be 1.54 per cent, a content only slightly greater than that isolated by Hopkins and Cole and less than that isolated by Dakin.

This tryptophane method has received much adverse criticism,

[88] Komm, E., and Böhringer, E., *Z. physiol. Chem.*, 1923, cxxiv 287.
[89] Kraus, I., *J. Biol. Chem.*, 1925, lxiii, 157.
[90] Tillmans, J., and Alt, A., *Biochem. Z.*, 1925, clxiv, 135.
[91] Fürth, O., *Biochem. Z.*, 1926, clxix, 117.
[92] Tillmans, J., and Alt, A., *Biochem Z.*, 1926, clxxviii, 243.

especially from Gortner and Holm,[13] Fürth and his coworkers [81, 82] and Kraus.[89] Looney [21] answered several of these criticisms effectively and redetermined the tryptophane content of a number of proteins by the Folin and Looney method.

Recently Folin and Ciocalteu [22] have made a detailed study of the factors affecting the accuracy of the Folin and Looney methods for tyrosine and tryptophane. As an unforeseen result of this study, a new colorimetric method for tyrosine (page 145) was proposed. Two modified procedures for tryptophane were developed, and a number of minor changes in the Folin and Looney technic were proposed. Hydrolysis of the protein with fairly strong sodium hydroxide instead of baryta was suggested; the phenol reagent was modified to contain lithium sulfate in order to obtain clear solutions showing true proportionality in color; and tyrosine standards were suggested in place of pure tryptophane standards, because pure tryptophane is expensive, and also because the tryptophane solutions deteriorate.

According to Folin and Ciocalteu a wide range of true proportionality between different amounts of tyrosine and tryptophane and the phenol reagent cannot be obtained unless the reagents alone produce no color under the conditions employed. Colorless controls, unfortunately, are not always attained under the conditions prescribed by Folin and Looney for the determination of tryptophane. The cause of the color was traced to the cyanide, concerning which Folin and Ciocalteu said, "The more we have worked with sodium cyanide the more doubtful have we become as to the wisdom of retaining it in these particular methods. The trouble with the cyanide is not only that the best grades give a color with the phenol reagent, but the poorer grades give much more, and will thus prove an incalculable source of error. . . . One particularly treacherous feature of the cyanide in this connection is that the blue color which it gives with the phenol reagent alone is no criterion as to the amount of color it will give in the presence of a mercuric salt." Folin and Ciocalteu conclude, however, "Notwithstanding these serious drawbacks to the cyanide, it is possible to use it and obtain excellent analytical results; but as it stands, the method probably can never become a standard method, because it does not adequately represent the well nigh perfect character of the underlying color reaction."

The two modifications proposed by Folin and Ciocalteu for the determination of tryptophane differ from the Folin and Looney method mainly in the way in which the mercuric sulfate precipitate of tryptophane is decomposed. In the Folin and Looney procedure sodium cyanide was used. In the first new procedure the mercury-tryptophane compound

is decomposed by means of hydrogen sulfide in acid solution and in the second, hot strong hydrochloric acid is used. The tryptophane content of casein as reported by Folin and Ciocalteu was 1.4 per cent, a figure still lower than that reported by Folin and Looney.

Onslow's Differential Nitrogen Method

In 1921 Onslow [93] investigated the nature of the substances precipitated by mercuric sulfate from casein digested with enzymes. He found, besides tryptophane, leucine, cystine, glutamic acid, aspartic acid, histidine and a little proline. In 1924 Onslow [94] proposed a method for the determination of tryptophane based on these findings. On the assumption that the total nitrogen of a mercuric sulfate precipitate is given by the following equation:

Total N = Tryptophane N + Histidine N + Monoamino N + Peptide N,

tryptophane N may be calculated as follows:

Tryptophane N = 2(Monoamino N — (⅔ histidine N) + peptide non-amino N).

The tryptophane nitrogen is obtained indirectly from this equation by evaluating the three unknown quantities in the right hand member, as follows: The non-amino nitrogen is the difference between the total nitrogen as determined by the Kjeldahl method and the amino nitrogen as obtained by Van Slyke's method; histidine is estimated by the method of Koessler and Hanke,[35] and the peptide non-amino nitrogen is the difference between the amino nitrogen before and after complete hydrolysis. With proteins containing large amounts of proline, special precautions are recommended; and, similarly, with proteins containing large amounts of cystine, an estimation of the total sulfur and a correction due to the cystine nitrogen is necessary.

By this method Onslow found an average of 1.93 per cent tryptophane in English casein and 2.15 per cent in Merck's casein.

Summary

From the above brief consideration of the methods proposed for the quantitative determination of tryptophane in proteins, it would indeed be hazardous to recommend any one method. For purposes of comparison the analysis of casein as obtained by each method has been summarized in the following table.

[93] Onslow, H., *Biochem. J.*, 1921, xv, 392.
[94] Onslow, H., *Biochem. J.*, 1924, xviii, 63.

TABLE 7. *The Tryptophane Content of Casein as Determined by Different Methods.*

Investigator and Reference Number	Year	Tryptophane *Per Cent*	Method
Hopkins and Cole (45)	1901–02	1.5	Isolated and weighed
Dakin (66)	1918	1.7	Isolated and weighed
Fasal (69)	1912	0.65	Colorimetric (glyoxylic acid reaction)
Sanders and May (71)	1912–13	1.6	From indole produced by bacteria
Moraczewski (72)	1913	0.6	From indole produced by bacteria
Herzfeld (74)	1913	0.5	Colorimetric (*p*-dimethylaminobenzaldehyde)
Thomas (95)	1914	1.75	Colorimetric (*p*-dimethylaminobenzaldehyde)
Thomas (76)	1920	1.7–1.8	Colorimetric (*p*-dimethylaminobenzaldehyde)
Holm and Greenbank (62)	1923	2.2	Colorimetric (*p*-dimethylaminobenzaldehyde)
Jones, Gersdorff and Moeller (56)	1924	2.2	Colorimetric (*p*-dimethylaminobenzaldehyde)
Homer (79)	1915	1.0–1.6	Bromine titration
Fürth and Nobel (81)	1920	1.9–2.26	Colorimetric (Voisenet's aldehyde reaction)
Fürth and Lieben (82)	1920	2.0	Colorimetric (Voisenet's aldehyde reaction)
Lüscher (85)	1922	1.1–1.4	Colorimetric (Voisenet's aldehyde reaction)
Komm and Böhringer (88)	1923	2.2–2.3	Colorimetric (Voisenet's aldehyde reaction)
Fürth and Dische (83)	1924	1.7	Colorimetric (Voisenet's aldehyde reaction)
Tillmans and Alt (90)	1925	1.6	Colorimetric (Voisenet's aldehyde reaction)
Folin and Looney (20)	1922	1.54	Colorimetric (phenol reagent)
Folin and Ciocalteu (22)	1927	1.4	Colorimetric (phenol reagent)
Onslow (94)	1924	1.9–2.15	Differential nitrogen

Hopkins and Cole, the discoverers of tryptophane, actually isolated 1.5 per cent tryptophane from casein. Dakin, moreover, by a combination of the Hopkins and Cole method with his butyl alcohol extraction, isolated 1.7 per cent and Onslow,[93] using Dakin's method, claims that this value may be obtained in routine work. It is, therefore, reasonable to suppose that the tryptophane content of casein must be at least 1.7 per cent. The accuracy of methods which are not capable of giving a tryptophane value to casein of at least 1.7 per cent is to be looked

[93] Thomas, P., *Bull. soc. chim. biol.*, 1914, i, 67.

upon with suspicion. In this connection a quotation from Looney [21] is pertinent: "The multiplicity of methods proposed for the estimation of tryptophane and the inconsistency of the values given are sufficient evidence of the importance attached to the subject and the worthlessness of most of the methods."

ARGININE

While arginine is usually determined along with histidine and lysine by the Van Slyke method, the Kossel and Kutscher method, or by one of the modifications of the latter method, and among these the recent procedure of Vickery and Leavenworth [96] is especially noteworthy, there have been attempts to estimate arginine alone.

The Determination of Arginine with Permanganate

Orglmeister [97] in 1906 proposed a method for the determination of arginine with permanganate. The protein was hydrolyzed and the hydrolysate oxidized with calcium permanganate. The guanidine thus formed was precipitated as the picrate and its total nitrogen content used as an indication of the arginine content of the protein. This method assumes that during such treatment of a protein hydrolysate arginine only is oxidized to produce guanidine. This assumption, however, has been questioned.[98]

Determination of Arginine by Means of Arginase

Perhaps the simplest means of estimating arginine alone is by the use of arginase. Under suitable conditions arginase will bring about a practically quantitative conversion of arginine into ornithine and urea. The first to make use of arginase as a means of quantitative arginine determinations was Jansen [99] in 1917, who was seeking a method more accurate than either the Van Slyke or the Kossel method for studying the relationship between arginine and creatinine in the animal body. In Jansen's method albumin was hydrolyzed with concentrated hydrochloric acid, the hydrochloric acid evaporated off, the concentrated solution diluted with water, and then made neutral to neutral red. One portion of this solution was treated with urease and two portions with arginase plus urease. After 24 hours the ammonia was distilled into standard acid. Subtracting the ammonia found in the portion treated with urease alone from the total found in the portions treated with arginase and urease gave the ammonia due to arginine.

[96] Vickery, H. B., and Leavenworth, C. S. *J. Biol. Chem.*, 1928, lxxvi, 707.
[97] Orglmeister, G., *Beitr. chem. Physiol. Pathol.*, 1906, vii, 21.
[98] Otori, J., *Z. physiol. Chem.*, 1904, xliii, 86.
[99] Jansen, B. C. P., *Chem. Weekblad*, 1917, xiv, 125.

Hunter and Morrell [100] determined the optimum conditions for the action of arginase to be a reaction of pH 7 and a temperature of 50°. Hunter and Dauphinee,[101] using a method similar to that of Jansen, determined the arginine in the solution of the bases obtained in the Van Slyke method of protein analysis. Their figures, after correcting for the solubility of the arginine phosphotungstate according to Van Slyke, agreed closely with those obtained by the Van Slyke alkaline hydrolysis procedure.

A recent modification of Jansen's method and one claimed to have an average error of less than 2 per cent has been described by Bonot and Cahn.[102] A small amount (3 grams) of pure protein is hydrolyzed with 20 per cent hydrochloric acid for 48 hours, the acid is removed by concentration, and the solution is decolorized and brought to a reaction of pH 9.9. The solution is then treated with an excess of arginase for 72 hours at 37° C. The large amount of monoamino acids usually present suffices as a buffer to maintain the hydrogen ion concentration at its original value. After completion of the reaction the solution is neutralized, filtered, concentrated, taken up in 70 per cent acetic acid, clarified, and the urea precipitated with xanthydrol in methyl alcohol. The dixanthydrol urea is filtered, washed, dried and weighed. Multiplying the figure for the urea by 0.414 gives the weight of arginine.

Determination of Arginine by Means of Flavianic Acid

Kossel and Gross [103, 104] found that flavianic acid (1-naphthol-2, 4-dinitro-7-sulfonic acid) forms crystalline compounds with organic bases, and that the arginine compound is comparatively insoluble. It was further shown that this salt can be used in the quantitative estimation of arginine in protein hydrolysates. The protein is hydrolyzed with sulfuric acid, most of the acid removed as its barium salt, and a concentrated solution of flavianic acid (approximately 4 grams of the acid for each gram of arginine expected) added. After standing for three days in a cool place, the precipitate is filtered off, washed, recrystallized from water, and weighed. Arginine is calculated from the weight of the arginine flavianate.*

Recently Kossel and Staudt [105] described a modification of this

[100] Hunter, A., and Morrell, J. A., *Trans. Roy. Soc. (Canada)*, 1922, xvi, Sect. V, 75.
[101] Hunter, A., and Dauphinee, J. A., *J. Biol. Chem.*, 1925, lxiii, 11.
[102] Bonot, A., and Cahn, T., *Compt. rend. acad. sci.*, 1927, clxxxiv, 246.
[103] Kossel, A., and Gross R. E., *Sitzb. Heidelberg Akad. Wiss.*, Abt. B. i, 1; cited through *Chem. Abstr.*, 1924, xviii, 1681.
[104] Kossel, A., and Gross, R. E., *Z. physiol. Chem.*, 1924, cxxxv, 167.
* Pratt (Pratt, A. E., *J. Biol. Chem.*, 1926 lxvii, 231) found considerable losses of arginine to occur in this procedure and suggested modifications which made the process especially applicable to the preparation of arginine.
[105] Kossel, A., and Staudt, W., *Z. physiol. Chem.*, 1926, clvi, 270.

method so as to make it applicable to the determination of histidine as well as arginine. It was claimed that arginine was quantitatively precipitated as the flavianate at hydrogen ion concentrations between the turning point of litmus and that of 0.1 *N* sulfuric acid. The presence of an equal amount of histidine was claimed not to interfere. Arginine was thus determined as the flavianate. The arginine nitrogen subtracted from the total nitrogen of the silver nitrate-barium hydrate precipitate, as usually obtained in the many modifications of the Kossel and Kutscher method for the determination of the hexone bases, gave the histidine nitrogen. Vickery and Leavenworth,[96] who make use of flavianic acid in a recent modification of the Kossel method for the determination of arginine, histidine and lysine, claim the most complete separation of arginine silver and histidine silver is made at a reaction of pH 7.0, previous to the precipitation of arginine as the flavianate.

Direct Estimation of Arginine by Alkali

In the Van Slyke method of protein analysis, arginine is determined by boiling the solution of the bases with strong alkali. Arginine when boiled with alkali decomposes into ornithine and urea and the urea into ammonia. Unless considerable cystine, which also is decomposed to some extent under these conditions, producing ammonia, is present the ammonia nitrogen evolved represents one-half of the arginine nitrogen. Plimmer and Rosedale [106] treated a protein hydrolysate directly with alkali in the same manner as was recommended by Plimmer [107] for the determination of arginine in the solution of the bases in the Van Slyke procedure. On the assumption that no other amino acid, except arginine and cystine, gave off ammonia under these conditions, the ammonia produced (corrected if necessary for that produced by cystine) was a measure of the arginine in the protein.

Plimmer and Rosedale found that arginine, estimated directly in the protein hydrolysate, had a higher value than if estimated in the diamino fraction precipitated by phosphotungstic acid. On treating the monoamino fraction in the same manner, a value was obtained which represented the difference. They prefer the arginine value obtained directly from the protein hydrolysate as representing more accurately the arginine content of the protein.

HISTIDINE

The first method for the determination of histidine to give approximately quantitative results was that of Kossel and Kutscher, who in

[106] Plimmer, R. H. A., and Rosedale, J. L., *Biochem. J.*, 1925, xix, 1020.
[107] Plimmer, R. H. A., *Biochem. J.*, 1916, x, 115.

1900 proposed their original method for the separate determination of arginine, histidine, and lysine. Modifications of this method have appeared from time to time, until now these three amino acids may be determined with comparative ease and with an excellent degree of accuracy. These methods have been described in the previous chapter. Some attempts, however, have been made to estimate the histidine content of proteins without regard to any other constituent.

The Copper Method of Kober and Sugiura

In 1913 Kober and Sugiura [108] proposed a method for the quantitative estimation of histidine. The method for histidine was a modification of their method for the quantitative determination of total amino acids, based on the property of the amino acids of reacting quantitatively with cupric hydroxide to form copper complexes. Their method for histidine depended upon the property of normal caproic acid of precipitating the copper quantitatively from all the amino acids with the exception of histidine. The insoluble histidine-copper complex was then dissolved in warm acetic acid and titrated iodometrically. Onslow,[94] however, tried to use this method, but did not find it as specific as had been claimed.

Colorimetric Estimation of Histidine

In 1913 Weiss and Ssobalew [36] proposed a method for the quantitative estimation of histidine based on Pauly's [109] diazo colorimetric test for this amino acid. Histidine, and also tyrosine, produce a red color when treated with *p*-phenyldiazonium sulfonate (*p*-diazobenzene sulfonate). In the Weiss and Ssobalew procedure the histidine is first isolated according to the method of Kossel. The reagents used are a solution of 1 per cent sulfanilic acid in hydrochloric acid and a 0.5 per cent solution of sodium nitrite. A mixture of these is added to the solution to be tested and then the mixture is treated with a solution of sodium carbonate. The color produced is compared with that developed in a similar manner from a standard histidine solution. In the application of the method to protein hydrolysates certain inhibitory substances sometimes are present and seem to combine with the diazonium compound. A scheme is suggested by means of which the influence of these inhibitory substances is removed by carrying out the reaction systematically in the presence of varying amounts of the reagents.

Lautenschläger [110] also proposed a method for the determination of histidine based upon its reaction with *p*-diazobenzene sulfonate. The

[108] Kober, P. A., and Sugiura, K., *J. Am. Chem. Soc.*, 1913, xxxv, 1546.
[109] Pauly, H., *Z. physiol. Chem.*, 1904, xlii, 508.
[110] Lautenschläger, C. L., *Z. physiol.*, 1918, cii, 226.

histidine is separated from tyrosine either by precipitation with silver nitrate in neutral solution or by mercuric chloride in alkaline solution. The histidine solution is then treated with an excess of standard *p*-diazobenzene sulfonic acid solution, the solution made acid with hydrochloric acid, an excess of standard titanium trichloride added to reduce the excess of the diazobenzene sulfonic acid, and then the excess of titanium trichloride determined by titration with ferric alum solution using a thiocyanate as indicator. However, Onslow [94] made an exhaustive test of this method and found that under the conditions described the diazobenzene sulfonic acid did not react quantitatively.

Next to make use of *p*-diazobenzene sulfonate as a reagent for the quantitative estimation of imidazole derivatives were Koessler and Hanke.[35] This method was made applicable to the determination of histidine in proteins by Hanke and Koessler [111] in the following manner: The protein is hydrolyzed with hydrochloric acid, the acid removed by vacuum distillation, and the ammonia and humin removed with lime in the usual manner. Histidine, together with arginine, lysine, and cystine, are precipitated with phosphotungstic acid. Of these four amino acids, histidine is the only one that gives an orange-red color with alkaline *p*-diazobenzene sulfonate. The phosphotungstic acid precipitate is treated with a solution of sodium hydroxide and the histidine in the resulting quantitative estimation of imidazole derivatives were Koessler and Hanke. The standard used is a solution of Congo red and methyl orange.

In 1925 Hanke [34] described, for the quantitative determination of histidine and tyrosine, a method in which the procedure for histidine is slightly modified from that just described. The protein (1 to 3 grams) is hydrolyzed with sulfuric acid, the sulfuric acid removed with barium, and the histidine precipitated with silver nitrate and barium hydroxide as in the usual Kossel method. The histidine silver compound is decomposed by the use of hydrochloric acid, and the histidine determined colorimetrically by means of diazobenzene sulfonate as before. Results obtained by this method are comparable with those obtained by the phosphotungstic acid procedure.

The Determination of Histidine by Bromination

In 1908 Knoop [112] described a color reaction for histidine with bromine water in which bromine was apparently absorbed. Thrun and Trowbridge [113] in 1918 proposed a method for the estimation of histidine in proteins by brominating the solution of the bases as obtained in the

[111] Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1920, xliii, 527.
[112] Knoop, F., *Beitr. chem. Physiol. Pathol.*, 1908, xi. 356.
[113] Thrun, W. E., and Trowbridge, P. F., *J. Biol. Chem.*, 1918, xxxiv, 355.

Van Slyke procedure. Corrections were made for the bromine absorbed by cystine and for the solubility of the histidine phosphotungstate. The results agreed well with those obtained by the Van Slyke method. These authors later [114] described a similar procedure applicable to the determination of histidine in proteins without the use of the Van Slyke procedure.

Plimmer and Phillips [28] have described a method for the estimation of histidine and tyrosine in proteins, the histidine procedure being much like that first described by Thrun and Trowbridge. The estimation of histidine depends upon the assumption that it reacts quantitatively with two atoms of bromine and that arginine and lysine do not absorb bromine. The products of decomposition of tryptophane, which are formed during acid hydrolysis and which absorb bromine, are assumed to be completely contained in the humin precipitated by lime. Tyrosine does not interfere, since this is separated from histidine by means of phosphotungstic acid. The presence of cystine was thought not to cause serious error unless present in large quantities in the original protein. The bromination of histidine is carried out according to the procedure described by Plimmer and Eaves [27] for the bromination of tyrosine, in which an excess of standard sodium bromate and potassium bromide solutions is added to the solution of the amino acid in dilute hydrochloric acid. After an interval of ten to fifteen minutes an excess of potassium iodide solution is introduced and the liberated iodine, corresponding to the excess of bromine, is titrated with standard sodium thiosulfate solution.

Titration of Histidine with Standard Acid

A method which promises to be of considerable value in the study of the composition of protein hydrolysates is the titration method of Harris. From a theoretical consideration of the factors involved in the titration of the amino and carboxyl groups of the amino acids,[115] Harris [116, 117] recommends the use of the quinhydrone electrode as a means of determining amino or other basic or acidic groups in an amino acid mixture. He describes methods for the estimation (*a*) of an amino acid by titrating the amino group, (*b*) of the total amino groups, (*c*) of lysine and arginine or of histidine, alone or in a mixture of "neutral" amino acids, and (*d*) glutamic acid and aspartic acids, alone or in a mixture of "neutral" amino acids, by titration of the stronger carboxyl

[114] Thrun, W. E., and Trowbridge, P. F., *J. Assoc. Off. Agr. Chem.*, 1920, iv, 194.
[115] Harris, L. J., *Proc. Roy. Soc. (London)*, 1923-24, B, xcv, 500.
[116] Harris, L. J., *Proc. Roy. Soc. (London)*, 1923-24, B, xcv, 440.
[117] Harris, L. J., *J. Chem. Soc.*, 1923, cxxiii, 3294.

group. Concerning the estimation of histidine, he says, "The determination of the concentration of histidine in solution is found to be a particularly simple operation, because the end-point (99 per cent neutralization) for K_{b1} occurs at about pH 3.76, where the secondary effect of the HCl on the solvent plays no appreciable part. Hence after the addition of one mol of HCl to one mol of histidine, there occurs a very great increase of hydrogen ion concentration for a very slight addition of acid. In other words, the uncorrected titration curve becomes approximately vertical with the completion of the titration. There is, therefore, produced a sharp change in the color of an indicator having a transition point near pH 3.76. In consequence, histidine may be estimated with an ease and accuracy almost equal to that of the titration of a strong alkali with standard acid; and this in spite of the fact that an aqueous solution of histidine has a pH not far removed from that of pure water."

The Method of Kossel and Staudt

In the previous section on arginine, there was described a method by Kossel and Staudt [105] in which arginine could be quantitatively separated from the other amino acids in a protein hydrolysate by means of flavianic acid. After the determination of arginine as the flavianate, the difference between the arginine nitrogen and the total nitrogen of a silver nitrate-barium hydroxide precipitate, as obtained in the usual Kossel procedure, represents the histidine nitrogen.

GLUTAMIC AND ASPARTIC ACIDS

Isolation of Glutamic Acid as the Hydrochloride

Glutamic acid has long been isolated from protein hydrolysates as its hydrochloride. Hlasiwetz and Habermann [118] in 1873 introduced the method and it is still a customary procedure preliminary to a Fischer esterification. The protein is hydrolyzed with hydrochloric acid, filtered, and concentrated *in vacuo* to one-half or one-third its volume. The solution is then saturated with dry gaseous hydrochloric acid and allowed to stand at 0° for several days; glutamic acid hydrochloride crystallizes out. The precipitate is filtered off, washed with ice-cold alcoholic hydrochloric acid, and redissolved in water, and the solution is decolorized, and then freed from ammonia by boiling with baryta. The barium is removed with sulfuric acid and the glutamic acid is again precipitated as the hydrochloride. Further crops of crystals may usually

[118] Hlasiwetz, H., and Habermann, J., *Ann. Chem.*, 1873, clxix, 150.

be obtained from the mother liquors. All of the glutamic acid of the protein is not obtained by this means, but the precipitation is very nearly complete. The last traces of glutamic acid may be obtained as its ethyl ester during the Fischer esterification process.

Abderhalden [2] stated that the glutamic acid may be readily obtained from the hydrochloride by dissolving in water, passing in ammonia, evaporating to dryness and recrystallizing from water.

Isolation of Glutamic Acid as the Zinc Salt

Plimmer [3, p. 34] describes a procedure, to be used in place of the hydrochloride method especially in cases of proteins containing less than 10 per cent of glutamic acid, based on the observation of Kutscher [119] that glutamic acid forms a very insoluble zinc salt. The mixture of the amino acids, after the removal of most of the hydrochloric acid, is diluted with water, boiled with an excess of zinc oxide, and allowed to cool. The precipitate of the excess of zinc oxide and zinc glutamate is dissolved in hot acetic acid and decomposed with hydrogen sulfide. On evaporation glutamic acid remains.

A disadvantage of this method is that leucine also forms an insoluble zinc salt. Osborne and Liddle,[120] however, effect a separation of glutamic acid and leucine by neutralizing the acidity of the latter and crystallizing out the leucine. The glutamic acid is then obtained either by acidifying and crystallizing or as the hydrochloride.

Isolation of Glutamic and Aspartic Acids as Silver Salts

Kutscher [119] has proposed a method for the isolation of aspartic and glutamic acids depending upon the insolubility of their silver salts.[121] The mixture of amino acids, freed from the diamino acids and hydrochloric acid, if this acid has been used, is treated with a soluble silver salt and baryta, carefully avoiding an excess of the latter. The silver salts are decomposed and the aspartic acid separated from the glutamic acid either by the formation of the glutamic acid hydrochloride or by preparing the zinc salts; the zinc salt of aspartic acid is readily soluble in water.

Isolation of Glutamic and Aspartic Acids as Calcium Salts

Perhaps the most useful method yet proposed for the separation of aspartic and glutamic acids from the mixture of amino acids resulting

[119] Kutscher, F., *Z. physiol. Chem.*, 1903, xxxviii, 111.
[120] Osborne, T. B., and Liddle, L. M., *Am. J. Physiol.*, 1910, xxvi, 420.
[121] Habermann, J., *Ann. Chem.*, 1875, clxxix, 248.

from the hydrolysis of a protein is that of Foreman,[122] who found that the calcium salts of these amino acids were insoluble in alcohol. According to this procedure the hydrochloric acid solution of the amino acids is evaporated to a syrup *in vacuo,* the syrup dissolved in water and 0.5 gram of slaked calcium oxide added for each gram of protein taken. After shaking, the excess lime and the humin substances are filtered off. The filtrate is then concentrated *in vacuo* to remove the ammonia. Alcohol is now slowly added with shaking as long as a precipitate continues to form, after which the precipitate is filtered off and washed with alcohol. The precipitate is taken up with water and the calcium removed as the oxalate. By separate treatments with silver sulfate and phosphotungstic acid the solution is freed from all contaminating substances and on evaporation the aspartic and glutamic acids crystallize out. These are finally washed twice with cold glacial acetic acid, dried, and weighed.

The residues of glutamic and aspartic acids are separated, the glutamic acid as the hydrochloride and aspartic acid as copper aspartate. The proportion of each acid in the mixture of the two may also be calculated from the carbon content of the mixture. The proportion of aspartic acid in the mixture is given by multiplying the yield by

$$\frac{40.82 - \text{per cent of carbon}}{40.82 - 36.09},$$

the remainder being glutamic acid.

It was by the use of this method, combined with his butyl extraction method, that Dakin [66] discovered β-hydroxyglutamic acid. For the separation and determination of glutamic acid and of aspartic acid, Dakin [66] simplified the Foreman procedure somewhat. He isolates the glutamic acid as the hydrochloride, while the aspartic acid, after its preliminary precipitation with lead hydroxide as suggested by Levene and Van Slyke,[123] is isolated as its copper salt.

Determination of Glutamic and Aspartic Acids in the Van Slyke Analytical Procedure

Anderson and Roed-Müller [124] have proposed a modification of the Van Slyke method of protein analysis, making it applicable to the determination of the monoamino-dicarboxylic amino acids in addition to the usual arginine, histidine, lysine and cystine. The procedure is completely described by Plimmer.[3, p. 105-6] An aliquot of the solution of the

[122] Foreman, F. W., *Biochem. J.,* 1914, viii, 463.
[123] Levene, P. A., and Van Slyke, D. D., *J. Biol. Chem.,* 1910, viii, 285.
[124] Anderson, A. C., and Roed-Müller, R., *Biochem. Z.,* 1916, lxxiii, 326.

"filtrate from the bases," prepared in a manner only slightly different from that described by Van Slyke, is carefully neutralized evaporated to dryness and ignited. The ash is treated with a slight excess of standard acid and the excess acid titrated with standard alkali. The method is based on the assumption that, under the conditions employed, only the carboxyl groups of the monoamino-dicarboxylic acids are titrated, all monoamino-monocarboxylic acids having no effect.

Using recent modifications, especially those for aspartic acid, Jones and Moeller [125] have determined and reported the glutamic acid and aspartic acid content of a number of proteins.*

PROLINE

Proline was originally determined in the routine Fischer esterification procedure by extracting with alcohol that fraction of the amino acids whose esters boil below 90° at less than 1 mm. pressure. This method was based on the assumption that in this fraction proline was the only amino acid soluble in alcohol. However, the values of proline determined in this manner are always too high, due to slight solubilities of other amino acids. Purification of the impure proline in the alcoholic extract by racemization and recrystallization as the *d-l*-copper salt was not entirely satisfactory because of the tendency of other amino acids to form insoluble copper salts also.

An accurate method for the determination of proline is considered to be that of Van Slyke,[126] who makes use of the fact that proline evolves none of its nitrogen when treated with nitrous acid. On the contrary, all of the other amino acids, which might be found in the alcoholic extract of that fraction of amino acids whose esters boil below 90° at less than 1 mm. pressure, give up their nitrogen completely on similar treatment. Thus, proline can be accurately determined by making, on this alcohol soluble portion, a total nitrogen determination by the Kjeldahl method and subtracting from it the amino nitrogen as determined by the nitrous acid method of Van Slyke,[127] the difference being proline nitrogen.

By the use of this method, Van Slyke obtained a proline content of casein of 6.7 per cent, a figure twice that obtained by the original Fischer

[125] Jones, D. B., and Moeller, O., *Proc. Am. Soc., Biol. Chem.*, 1927, xxi, 54.

* Jones and Moeller (*J. Biol. Chem.*, 1928, lxxix, 429) have recently reported the aspartic and glutanic acid content of some thirteen proteins. The procedure used was based on the principle discovered by Foreman, that the dicarboxylic amino acids could be practically quantitatively separated from most of the other products in protein hydrolysates by precipitation of their calcium salts with alcohol. Barium instead of calcium saits were employed, however, mainly because of the ease with which this metal could be removed. Glutamic acid was separated as the hydrochloride and the aspartic acid as the copper salt.

[126] Van Slyke, D. D., *J. Biol. Chem.*, 1911, ix, 205.

[127] Van Slyke, D. D., *J. Biol. Chem.*, 1911, ix, 185.

method. Engeland,[128] by the use of his exhaustive methylation method (see p. 117), also reported 6.7 per cent proline in casein. Engeland's method, though, is nearly as long and tedious as Fischer's.

Proline can be determined quite readily and accurately by Dakin's [66] butyl alcohol method, especially Dakin's [129] modification involving extraction under reduced pressure. Proline is readily extracted by butyl alcohol and is readily soluble in alcohol. For the determination of proline, at the end of the extraction the butyl alcohol is filtered to remove suspended amino acids, the alcohol evaporated under diminished pressure, and the syrup taken up in absolute alcohol. This procedure is repeated once. Dakin claims that purification of the proline obtained in this manner is of negligible value. A small quantity of impurities is removed by a treatment with mercuric acetate and baryta and the proline finally dried under reduced pressure. The yield is calculated from a determination of the non-amino nitrogen, as suggested by Van Slyke, in preference to direct weighing of the hygroscopic compound. Dakin reported 8.0 per cent proline in casein [66] and 9.5 per cent in gelatin.[129]

OTHER AMINO ACIDS

With the possible exception of glycine, whose ethyl ester hydrochloride can be made to crystallize almost quantitatively from a mixture of the remaining amino acid ethyl ester hydrochlorides by concentrating *in vacuo* and allowing to stand at 0°,[130] there are no methods available for the quantitative determination of the other amino acids. Most of our knowledge concerning these amino acids has been obtained through the application of the Fischer esterification process or by one of its modifications, especially in the hands of Abderhalden, Osborne and his coworkers, Levene and Van Slyke, and Jones and Johns.

Perhaps the most valuable aid in the study of these amino acids —glycine, alanine, leucine, phenylalanine, isoleucine, valine, serine, hydroxyproline and hydroxyglutamic acid—will be Dakin's butyl alcohol extraction method, by which he has already recovered, as individual products, 91.31 per cent of the gelatin molecule and 101 per cent of zein.[131]

Other promising contributions are the titrimetric studies of Harris,[115, 116, 117] the use of electrolysis as recently described by Foster and Schmidt,[132, 133] and the carbamate method of Kingston and Schryver.[134]

[128] Engeland, R., *Ber. chem. Ges.*, 1909, xlii, 2962.
[129] Dakin, H. D., *J. Biol. Chem.*, 1920, xliv, 499.
[130] Fischer, E., *Z. physiol. Chem.*, 1902, xxxv, 229.
[131] Dakin, H. D., *Z. physiol. Chem.*, 1923, cxxx, 159.
[132] Foster, G. L., and Schmidt, C. L. A., *Proc. Soc. Exp. Biol. Med.*, 1921-22, xix, 348.
[133] Foster, G. L., and Schmidt, C. L. A., *J. Biol. Chem.*, 1923, lvi, 545.
[134] Kingston, H. L., and Schryver, S. B., *Biochem. J.*, 1924, xviii, 1070.

RESULTS OF PROTEIN ANALYSIS

The Amino Acid Content of Proteins

Although methods hitherto proposed for the determination of the amino acid components of proteins are with few exceptions not ideal nor of generally recognized accuracy, it is important to tabulate the results obtained if only to permit tentative estimates of their relative values in animal nutrition, to predict supplementary relations between different protein-containing foods, and to explain, if only partially, the differences in the biological values of proteins and protein mixtures already demonstrated by animal experimentation. In Plimmer's monograph on the Chemical Constitution of the Proteins [3] will be found exhaustive tables of protein analyses by isolation methods and by the popular Van Slyke procedure for the distribution of protein nitrogen among certain more or less definite chemical groupings. These tables cover the work done up to 1916 or 1917 and need not be repeated here, at least to any great extent. Since that time, however, a large amount of work has been done with the Van Slyke method, the colorimetric methods for tyrosine, tryptophane, cystine and histidine have been developed and applied extensively, and some work has been done also with gravimetric isolation methods. The data to be presented will be largely concerned with this recent work.

Two methods are in vogue for expressing analytical results obtained with proteins. According to one method, the amino acids are expressed as percentages of the ash- and moisture-free protein. This is a rational chemical method, although it will give high results unless the water added during hydrolysis is allowed for. The results are of significance primarily to the chemist and have been used to advantage in estimating the molecular weights of proteins.

In the field of nutritional physiology, however, protein analyses thus expressed are of less significance, because the digestion and metabolism of proteins in the animal body and their value in covering the protein requirements of the animal are investigated necessarily by following the disposition of dietary nitrogen among the excretions of the body and the retention of dietary nitrogen in the tissues, under certain definite conditions of feeding. The results of such investigations, although generally referred to a conventional protein (Nx6.25), are in reality coefficients of digestibility of *nitrogen,* biological values of digestible *nitrogen,* and *nitrogen* balances. In the interpretation of such results, or in predicting them from the nature of the diet, it would seem to be more appropriate to characterize chemically the sources of dietary

TABLE 8. *The Arginine, Histidine, Lysine, Tyrosine, Tryptophane and Cystine Content of the Proteins of Certain Cereal Seeds.**

Source	Character of Protein	Nitrogen Content	Percentage of Total Nitrogen in the Form of: Arginine	Histidine	Lysine	Tyrosine	Tryptophane	Cystine
I. Prolamins								
Oat		16.74	6.4[1]	2.3[1]	0.2[1]	...	0.0[10],[44]	2.4[10]
Wheat	Gliadin	17.66	5.4[2]	3.4[2], 3.2[47]	1.3[2]	1.4[8]	0.7[10], 0.8[45]	1.1[10]
Wheat	Gliadin	17.66	4.7[3]	4.3[3]	1.6[3]	1.3[46]	0.6[8],[44], 0.8[46]	1.6[46]
Wheat	Gliadin	17.66	5.0[13]	5.9[13]	0.6[13]	2.2[13]	0.9[13]	...
Barley	Hordein	16.92	5.4[4]	3.6[4], 1.6[47]	1.0[4]	...	0.6[10], 0.8[46]	1.0[10]
Rice		16.22	13.2[5]	6.2[5]	1.7[5]	...	...	1.3[5]
Rice		17.24	18.2[93]	8.1[93]	3.4[93]	...	...	0.9[93]
Corn	Zein	16.13	3.6[81]	1.3[6], 2.1[47]	0.0[6]	2.8[8], 2.7[46]	0.0[10],[44], 0.14[8]	0.5[46], 0.6[10]
Kafir	Kafirin	16.44	3.1[7]	1.8[7]	1.1[7]	...	0.8[10]	0.4[10]
Kafir	Kafirin	15.72	3.1[96]	1.6[96]	1.0[96]	...	...	0.5[96]
Wheat bran		15.56	9.1[9]	1.4[9]	3.0[9]	1.7[9]	1.2[9]	1.7[9]
II. Albumins								
Wheat bran		15.61	20.7[9]	4.4[9]	5.5[9]	2.1[9]	4.2[9]	2.5[9]
Barley	Leucosin	15.73	11.6[11]	4.5[11]	8.4[11]	...	1.2[44]	1.5[11]
III. Globulins								
Wheat bran		18.01	25.2[9]	4.1[9]	12.6[9]	1.6[9]	2.2[9]	1.0[9]
Oat	Avenalin	16.79	15.0[1]	8.3[1]	3.5[1]	...	0.9[44]	1.0[1]
Buckwheat		17.44	23.7[12]	0.9[12]	8.6[12]	...	2.1[10]	1.6[10]
Rice	Coagulated at 74°C.	16.31	15.5[19]	4.0[19]	8.4[19]	2.7[19]	2.2[19]	1.5[19]
Rice	Coagulated at 90°C.	17.94	27.2[19]	4.5[19]	3.9[19]	3.2[19]	1.8[19]	1.9[19]
Rice	Total globulins	17.04	23.5[92]	2.2[92]	7.7[92]	...	...	1.6[92]

TABLE 8. (*Continued*)

Source	Character of Protein	Nitrogen Content	Percentage of Total Nitrogen in the Form of: Arginine	Histidine	Lysine	Tyrosine	Tryptophane	Cystine
			IV. Glutelins					
Wheat	Glutenin	17.49	10.1[13]	8.5[13]	5.2[13]	2.5[13]	{1.2[13]} {1.3[46]}	1.2[46]
Wheat	Glutenin	17.49	9.5[14]	6.5[14]	2.2[14]	2.0[46]	1.3[10]	1.0[10]
Wheat	Glutenin	17.49	12.0[15]	4.7[15]	4.5[15]	...	{1.2[44]} {1.4[45]}	...
Wheat	Glutenin	16.64	10.9[16]	1.7[16]	5.8[16]	...	...	0.4[16]
Wheat	α-Glutelin	17.14	10.9[20]	5.5[20]	3.1[20]	...	...	1.8[20]
Wheat	β-Glutelin	16.06	6.1[20]	6.2[20]	6.8[20]	...	...	5.4[20]
Durum wheat		15.46	11.0[16]	5.8[16]	3.5[16]	...	...	0.9[16]
Oat	Avenin	15.84	14.4[1]	7.2[1]	4.4[1]	...	...	1.5[1]
Oat	Avenin	15.77	15.5[16]	3.2[16]	4.6[16]	...	...	1.1[16]
Oat	Avenin	17.53	15.3[17]	3.5[17]	5.4[17]	...	1.0[44]	2.0[17]
Rice	Oryzenin	16.91	16.4[18]	5.8[18]	7.3[18]	...	...	6.2[18]
Rice	Oryzenin	16.68	17.7[2]	5.4[2]	4.9[2]	...	...	0.9[2]
Rice	Oryzenin	16.41	17.9[16]	4.6[16]	4.4[16]	...	...	0.6[16]
Rice	Oryzenin	17.57	20.4[21]	3.7[21]	5.2[21]	2.3[19]	2.0[19]	0.7[19]
Rice	Oryzenin	17.15	18.9[92]	9.7[92]	5.4[92]	...	...	1.2[92]
Rice	Oryzenin	16.38	23.3[93]	6.8[93]	4.1[93]	...	...	1.2[93]
Barley	Hordenin	15.62	11.1[16]	2.0[16]	5.3[16]	...	1.1[44]	1.8[16]
Rye	Secalenin	14.47	13.8[16]	4.7[16]	6.6[16]	...	1.2[44]	0.6[16]
Corn	Zeanin	14.49	9.4[16]	3.9[16]	4.5[16]	...	1.0[44]	1.0[16]
Spelt		12.21	13.4[16]	2.8[16]	7.8[16]	...	...	0.4[16]
Emmer		14.58	13.0[16]	4.8[16]	6.0[16]	...	...	0.7[16]

* NOTE: The nitrogen contents of the proteins in this and following tables were obtained whenever possible from the reports giving the Van Slyke analyses. In other cases, other sources of information, such as Osborne's monograph on the Vegetable Proteins and Abderhalden's Biochemisches Handlexicon were consulted. The references indicated are listed on pages 192 to 194.

TABLE 9. *The Arginine, Histidine, Lysine, Tyrosine, Tryptophane and Cystine Content of the Proteins of Certain Non-Cereal Seeds.**

Source	Character of Protein	Nitrogen Content	Percentage of Total Nitrogen in the Form of: Arginine	Histidine	Lysine	Tyrosine	Tryptophane	Cystine
			Proteins from Beans					
Adzuki	α-Globulin	15.60	11.2[22]	3.9[22]	10.2[22]	...	1.5[10]	1.3[10]
Adzuki	β-Globulin	16.57	13.6[22]	4.1[22]	9.7[22]	...	0.8[10]	0.3[10]
Lima	Albumin	14.24	13.0[23]	4.9[23]	8.0[23]	...	1.3[10]	0.9[10]
Lima	α-Globulin	15.56	11.7[23]	6.5[23]	9.7[23]	...	1.7[10]	1.2[10]
Lima	β-Globulin	14.80	11.0[23]	4.8[23]	11.0[23]	...	2.0[10]	0.6[10]
Navy	α-Globulin (conphaseolin)	15.65	14.1[25]	1.5[25]	13.1[25]	...	2.4[10]	1.1[10]
Navy	β-Globulin (phaseolin)	16.30	12.6[25]	3.9[25]	11.1[25]	...	0.7[45]	0.8[25]
Navy	β-Globulin (phaseolin)	15.99	12.3[24]	5.6[24]	9.5[21]	...	0.8[10]	0.4[10]a
Georgia velvet	Albumin	15.99	12.3[26]	1.4[26]	9.8[26]	...	2.1[10]	2.0[10]
Georgia velvet	α-Globulin	16.73	13.8[26]	2.0[26]	9.6[26]	...	1.8[10]	2.3[10]
Georgia velvet	β-Globulin	17.13	15.4[26]	5.3[26]	9.5[26]	...	0.0[10]	0.4[10]
Chinese velvet	Stizolobin, by dialysis	16.33	14.1[27]	3.8[27]	10.0[27]	...	1.1[10]	1.1[10]
Chinese velvet	Stizolobin, by $(NH_4)_2SO_4$ pptn.	16.33	13.2[27]	4.4[27]	9.3[27]	...	...	0.9[27]
Chinese velvet	Stizolobin, coagulation	15.94	12.8[27]	4.3[27]	10.2[27]	...	...	0.9[27]
Soy	Glycinin	16.94	15.3[28]	2.4[28]	10.3[28]	...	1.3[10],[45]	0.8[10],[28]
Soy	Soluble in dilute alkali	15.99	14.6[29]	5.9[29]	8.3[29]	...	...	1.0[29]
Soy	Soybean casein	....	13.1[101]	6.9[101]	9.0[101]	...	...	...
Soy	Frozen bean casein	....	15.1[101]	3.7[101]	8.4[101]	...	...	...
Mung	α-Globulin	15.67	10.5[30]	5.7[30]	7.5[30]	...	1.8[10]	1.2[10]
Mung	β-Globulin	16.83	14.5[30]	3.2[30]	10.6[30]	...	1.0[10]	0.3[10]
			Proteins from Nuts					
Pecan	Globulin	15.76	22.9[31]	3.7[31]	6.2[31]	...	...	0.8[31]
Pecan	Soluble in dilute alkali	13.60	23.0[32]	3.7[32]	5.6[32]	...	...	0.8[32]
Peanut	Soluble in dilute alkali	14.08	17.2[32]	2.7[32]	5.7[32]	...	...	0.8[32]
Peanut	Arachin	18.28	23.8[33]	2.8[33]	5.2[33]	...	0.7[10]	0.7[10]
Peanut	Conarachin	18.23	25.8[33]	2.7[33]	6.3[33]	...	1.6[10]	1.9[10]
Peanut	Total globulin	....	25.3[100]	5.0[100]	4.1[100]	...	1.0[10]	1.4[10]
Coconut	Soluble in dilute alkali	15.68	28.6[29]	5.2[29]	4.8[29]	...	...	0.8[29]
Coconut	Globulin	17.38	29.5[34]	3.7[34]	6.4[34]	...	1.0[10]	1.0[10]
Cohune	Globulin	17.82	30.9[35]	2.6[35]	7.9[35]	...	0.5[10]	1.4[10]

TABLE 9. (*Continued*)

Source	Character of Protein	Nitrogen Content	Percentage of Total Nitrogen in the Form of: Arginine	Histidine	Lysine	Tyrosine	Trypto-phane	Cystine
Proteins from Oil-Bearing Seeds								
Cotton	α-Globulin	18.22	22.9[36]	5.3[36]	4.1[36]	...	...	0.5[36]
Cotton	β-Globulin	17.77	23.9[36]	6.1[36]	4.4[36]	...	...	0.5[36]
Cotton	Pentose protein	12.64	23.0[36]	3.1[36]	8.5[36]	...	...	1.4[36]
Cotton	Total globulins	18.20	22.7[29]	6.2[29]	4.5[29]	...	1.9[10]	0.9[29]
Cotton	Soluble in 0.2% NaOH	15.98	23.5[29]	4.9[29]	5.1[29]	...	...	1.1[29]
Cotton	Soluble in 5% $Ba(OH)_2$	16.75	24.0[29]	5.5[29]	4.5[29]	...	...	1.2[29]
Rape	Globulin (colzalin)	17.04	16.4[37]	12.2[37]	2.5[37]	...	...	1.0[37]
Sesame	α-Globulin	18.44	26.3[38]	3.9[38]	5.6[38]	2.0[38]	2.1[38]	1.2[38]
Sesame	β-Globulin	17.57	28.1[38]	5.2[38]	4.3[38]	2.0[38]	2.1[38]	1.0[38]
Hemp	Edestin	18.69	27.2[68]	5.5[68]	3.8[68]	1.9[46]	1.1[45] 1.8[10]	0.6[10]
Hemp	Edestin	18.69	...	...	...	1.9[8]	1.1[8] 1.1[46]	0.5[46]
Proteins from Miscellaneous Seeds								
Tomato	α-Globulin	18.54	24.2[40]	1.7[40]	5.0[40]	...	0.8[10]	0.7[10]
Tomato	β-Globulin	16.04	21.4[40]	6.4[40]	7.6[40]	...	1.2[10]	0.8[10]
Cantaloupe	Globulin	18.42	28.4[41]	6.2[41]	3.4[41]	...	2.1[10]	0.8[10]
Cantaloupe	Glutelin	16.37	24.4[41]	4.5[41]	5.4[41]	...	2.7[10]	0.8[10]
Squash	Globulin	18.39	27.5[41]	6.7[41] / 3.3[47]	3.8[41]	...	2.2[10]	0.9[10]
Mangold	A-Globulin	16.81	20.5[42]	10.5[42]	1.5[42]	...	...	1.8[42]
Mangold	B-Globulin	16.23	27.2[42]	3.8[42]	2.6[42]	...	...	1.9[42]
Alfalfa	Soluble in dilute alkali	15.54	21.4[43]	5.4[43]	6.1[43]	...	...	1.0[43]

* List of references indicated will be found on pages 192 to 194.

[a] However, Sullivan (*Proc. Amer. Soc. Biol. Chem.*, 1928, xv), employing his own method for cystine, found little if any of this amino acid in phaseolin.

nitrogen in terms of nitrogen, rather than in terms of protein, whose nitrogen content may vary from 12 to 19 per cent. This the Van Slyke analytical method does and the results obtained during the last ten to twelve years will be now considered.

The analyses summarized in Tables 8 to 11 relate to the percentage of the total nitrogen of proteins existing in the form of arginine, histidine, lysine, tyrosine, tryptophane and cystine. The results for the first three amino acids have been obtained by the Van Slyke method with few exceptions, those for tyrosine and tryptophane by colorimetric methods, and the results for cystine by colorimetric methods wherever possible, otherwise by the Van Slyke method.

The prolamins from the cereal seeds are in general low in the basic amino acids, particularly in lysine and in tryptophane. The prolamin of rice is rather sharply distinguished from the other proteins of its class by its relatively high content of arginine and histidine nitrogen. Zein and kafirin are notably low in cystine.

The albumins and globulins, accounting for only a small percentage of the nitrogen of the cereal seeds, are less readily characterized, though as a class they appear to be better balanced with respect to the six amino acids under consideration than are the prolamins. The low lysine content of oat globulin in conjunction with that of oat prolamin, is worthy of note. The exceptionally low histidine content of buckwheat globulin may also be mentioned.

The cereal glutelins, which, with the prolamins, account for most of the protein in the cereal seeds, are also difficult to characterize relative to their amino acid contents. In general, the lysine content is only moderately high and the arginine content less than that of the albumins and globulins. The tryptophane content is also only moderate, so that these proteins cannot be considered effective supplements to the prolamins in so far as these amino acids are concerned.

In contrast to the cereal seeds, the beans possess proteins relatively rich in lysine. The globulins are the most important proteins in these seeds. Their content of arginine is uniformly moderate, and that of histidine from moderate to low. Several of them are excellent sources of tryptophane and cystine, while others are notably poor in these constituents. The low cystine contents of some of the proteins of the adzuki, lima, navy, Georgia velvet and mung beans are noteworthy, in view of the results of biological experiments with some of these beans pointing also to a cystine deficiency.

The proteins of nuts, in so far as available analyses indicate, are uniformly rich in arginine, moderately rich in lysine, but relatively poor in

TABLE 10. *The Arginine, Histidine, Lysine and Cystine Content of the Proteins of Green Forage Plants and Roots. All Van Slyke Determinations.*

Source	Character of Protein	Nitrogen Content	Percentage Total Nitrogen in Form of: Arginine	Histidine	Lysine	Cystine	Reference*
Alfalfa leaf	Cytoplasmic proteins[1]	15.76	15.3	3.1	10.0	0.8	59
Alfalfa leaf	Soluble in dilute alkali	13.60	11.0	6.3	5.3	0.8	60
Alfalfa leaf	Water-soluble: A	13.94	18.8	6.8	5.5	1.3	61
Alfalfa leaf	Water-soluble: B	12.72	17.6	7.5	5.3	1.3	61
Crimson clover leaf	Cytoplasmic proteins	11.00	17.3	7.9	4.5	1.2	61
Red clover leaf	Cytoplasmic proteins	11.65	13.4	14.4	3.1	0.8	61
Sainfoin leaf	Cytoplasmic proteins	10.73	12.1	12.5	9.5	0.7	61
Common vetch leaf	Cytoplasmic proteins: A	14.40	17.8	3.5	11.3	1.0	61
Common vetch leaf	Cytoplasmic proteins: B	....	12.0	4.4	7.8	0.7	61
Corn leaf	Cytoplasmic proteins	14.41	14.7	4.7	8.8	0.8	62
Spinach leaf	Cytoplasmic proteins[2] (spinacin)	16.25	13.8	3.9	9.6	1.3	63
Cabbage leaf	Coagulum	13.82	13.3	8.3	7.3	1.2	64
Kohl-rabi leaf	Coagulum	14.00	14.4	8.4	7.2	1.3	64
Marrow-stem kali leaf	Coagulum	14.09	14.7	8.0	7.8	1.3	64
Marrow-stem kali stem	Coagulum	12.22	15.1	7.6	6.9	1.0	64
Turnip leaf	Coagulum	14.92	13.7	8.6	7.5	1.0	64
Turnip root	Coagulum	13.67	15.2	7.2	8.9	1.0	64
Cabbage leaf	Soluble in alkali	....	9.4	11.5	5.7	1.3	64
Turnip leaf	Soluble in alkali	....	11.7	7.7	6.1	1.1	64
Mangold root	Proteins of juice: I	11.80	14.9	5.6	6.7	1.6	65
Mangold root	Proteins of juice: II	11.53	16.7	5.2	7.0	1.0	65
Mangold root	Proteins of juice: III	11.95	16.5	3.0	5.5	0.9	65
Cauliflower	Water-soluble, coagulable	....	12.3	2.7	10.7	0.7	66
Cauliflower	Alkali-soluble, coagulable	....	14.0	6.1	10.6	1.0	66
Carrot	Water-soluble	14.72	15.4	4.4	6.3	1.1	67
Parsnip	Water-soluble	12.12	10.8	11.5	7.5	0.9	67

* List of references will be found on pages 192 to 194.

[1] Contains 2.5[10] per cent of tryptophane nitrogen, and 0.7[10] per cent of cystine nitrogen, according to colorimetric methods.

[2] Contains 1.6[10] per cent of tryptophane nitrogen, and 1.9[10] per cent of cystine nitrogen, according to colorimetric methods.

TABLE 11. *The Arginine, Histidine, Lysine, Tyrosine, Tryptophane and Cystine Content of Various Animal Proteins.**

Source	Character of Proteins	Nitrogen Content	Percentage of Total Nitrogen in the Form of: Arginine	Histidine	Lysine	Tyrosine	Tryptophane	Cystine
Milk	Casein	15.62	7.4[48]	6.2[48]	10.3[48]	2.7[58]	1.9[10]	0.2[10]
Milk	Casein	15.62	...	4.5[47]	...	3.2[8]	1.5[44], 1.3[58]	...
Milk	Lactalbumin	15.49	7.2[2]	4.6[2]	12.2[2]	...	2.4[10], 2.1[45]	3.0[10]
.....	Gelatin	17.89	14.7[39]	4.5[39]	6.3[39]	0.0	0.0	0.1[10], 0.1[46]
.....	Gelatin	17.89	13.6[69]	0.3[69], 0.8[47]	12.1[69]	...	...	...
.....	Gelatin	17.89	15.7[70]	6.7[70]	9.8[70]	...	...	...
Blood	Fibrin	16.91	13.9[39]	4.8[39], 3.4[47]	11.5[39]	2.9[58]	3.6[10], 2.3[58]	2.6[10]
Blood (horse)	Hemoglobin [e]	16.86	7.8[49]	13.0[49]	11.1[49]	2.6[49]	2.1[49]	0.0[49]
Blood (ox)	Hemoglobin	....	7.7[39]	12.7[39]	10.9[39]	...	...[a]	0.0[39]
Muscle (ox)	Coagulable proteins	16.67	13.3[50]	5.9[50]	13.9[50]	...	1.0[10]	1.1[10]
Muscle (ox)	Coagulable proteins	....	13.3[51]	5.0[51]	11.2[51]	...	...	...
Muscle (ox)	Coagulable proteins	....	14.0[53]	4.2[53]	13.2[53]	...	...	...
Muscle (ox)	Water-soluble	....	10.5[53]	6.8[53]	13.6[53]	...	...	...
Muscle (sheep)	Coagulable proteins	....	15.0[51]	18.0[51]	4.3[51]	...	...	...
Muscle (pig)	Coagulable proteins	....	14.0[51]	7.0[51]	7.0[51]	...	...	...
Muscle (horse)	Coagulable proteins	....	14.9[51]	10.5[51]	11.6[51]	...	...	...
reast (chicken)	Coagulable proteins	....	10.0[51]	13.0[51]	2.0[51]	...	...	...
Leg (chicken)	Coagulable proteins	....	8.0[51]	7.0[51]	11.0[51]	...	...	...
Rabbit, male	Myosin from muscle	15.99	20.7[94]	7.2[94]	8.1[94]	...	...	1.1[94]
Rabbit, female	Myosin from muscle	16.26	19.1[94]	10.8[94]	6.1[94]	...	...	0.8[94]
Rabbit, male	Myogen from muscle	15.84	18.9[94]	7.8[94]	7.6[94]	...	...	1.5[94]
Rabbit, female	Myogen from muscle	15.91	17.2[94]	11.7[94]	6.4[94]	...	...	1.4[94]
Bull	Myosin from muscle	16.03	17.8[94]	3.0[94]	9.3[94]	...	1.4[44]	1.5[94]
Cow	Myosin from muscle	15.88	16.4[94]	7.2[94]	8.9[94]	...	...	1.4[94]
Bull	Myogen from muscle	15.39	17.3[94]	2.5[94]	9.8[94]	...	...	1.7[94]
Cow	Myogen from muscle	15.37	16.6[94]	4.1[94]	9.6[94]	...	...	1.4[94]

TABLE 11. (*Continued*)

Source	Character of Proteins	Nitrogen Content	Percentage of Total Nitrogen in the Form of: Arginine	Histidine	Lysine	Tyrosine	Tryptophane	Cystine
Cock	Myosin from muscle	16.80	17.9[94]	5.2[94]	10.0[94]	...	...	1.7[94]
Hen	Myosin from muscle	16.47	17.3[94]	6.7[94]	9.0[94]	...	...	1.6[94]
Cock	Myogen from muscle	15.52	18.1[94]	3.3[94]	10.3[94]	...	...	1.2[94]
Hen	Myogen from muscle	15.01	18.1[94]	6.6[94]	10.1[94]	...	...	1.2[94]
Shrimp muscle	Total proteins	16.88	19.5[52]	6.1[52]	8.6[52]	2.2[52]	1.0[52]	1.2[52]
Sardine	Protamine	18.35	27.8[54]	23.0[54]	5.5[54]	0.5[54]	0.6[54]	0.6[54]
Anchovy, male	Total muscle proteins	16.53	7.1[102]	4.5[102]	7.7[102]	...	...	...
Anchovy, female	Total muscle proteins	16.71	6.0[102]	7.9[102]	7.3[102]	...	...	...
Herring, male	Total muscle proteins	16.21	7.1[102]	3.5[102]	9.0[102]	...	...	...
Herring, female	Total muscle proteins	16.23	6.5[102]	6.8[102]	6.2[102]	...	...	...
Grouper, male	Total muscle proteins	16.98	6.7[102]	6.3[102]	5.4[102]	...	...	...
Grouper, female	Total muscle proteins	16.94	6.6[102]	7.3[102]	4.8[102]	...	...	...
Tunny, male	Total muscle proteins	16.44	7.9[102]	2.4[102]	9.9[102]	...	...	...
Tunny, female	Total muscle proteins	16.08	6.5[102]	3.0[102]	9.8[102]	...	...	...
Dog	Hair	....	15.3[39]	3.5[39]	5.4[39]	...	...	6.6[39]
Man	Hair	17.36	14.9[56]	2.5[56]	5.4[56]	...	...	6.1[56]d
Man	Hair	17.25	16.6[56]	4.0[56]	5.6[56]	...	...	6.7[56]
Man	Nails	16.76	15.9[56]	3.4[56]	6.0[56]	...	...	3.4[56]
Man	Nails	16.21	17.4[56]	2.1[56]	5.2[56]	...	...	3.2[56]
Egg	Yolk[b]	....	14.5[57]	3.1[57]	9.4[57]	...	1.1[44], 1.5[45]	...
Egg	White[c]	....	11.7[57]	0.2[57], 4.0[47]	10.1[57]	2.0[8]	1.1[44], 1.2[8]	...
Egg	Membrane	....	16.9[57]	4.3[57]	9.4[57]	...	...	...
Egg	Ovomucoid	....	10.9[57]	0.0[57]	11.7[57]	...	...	...
Thyroid, hog	Thyreoglobulin	15.58	16.4[108]	11.6[108]	4.3[108]	2.7[108]	1.9[108]	1.2[108]

* List of references indicated will be found on pages 192 to 194.

[a] See A. Hunter and H. Borsook, *Trans. Roy. Soc. Canada*, May, 1922.

[b] The tryptophane content of egg yolk is figured on the basis of vitellin, containing 16.38 per cent of nitrogen.

[c] The histidine[47], tyrosine, and tryptophane content of egg white refer to egg albumin containing 15.51 per cent of nitrogen.

[d] Wilson and Lewis (*J. Biol. Chem.*, 1927, lxxiii, 543) have recently obtained much higher values for the cystine content of human hair by the use of the Folin and Looney colorimetric method. From 29 samples they obtained an average cystine content of 18.6 per cent, equivalent to 14.1 per cent of the total nitrogen figured on an average N content of the hair of 15.37 per cent.

[e] Vickery and Leavenworth (*J. Biol. Chem.*, 1928, lxxix, 377) recently reported 6.40, 12.40, and 9.30 percentages of the total nitrogen of horse hemoglobin (containing 16.69 per cent total nitrogen) to be in the form of arginine, histidine, and lysine nitrogen, respectively.

histidine. The analyses for tryptophane are too few and too variable to warrant any general statements, while the cystine analyses do not indicate any marked inferiority in their content of this amino acid.

The proteins of the oil-bearing seeds appear to resemble those of nuts in their high arginine contents. Their contents of histidine and lysine are variable and frequently so low as to suggest biological deficiencies. The analytical data for the other amino acids are not notable, except the low cystine content of edestin, the frequently studied globulin of hemp seed.

The analytical results secured with a variety of miscellaneous seeds, included in Table 9, call for no comment, aside from the exceptionally low content of lysine in the two globulins or globulin fractions obtained from mangold seed.

Within recent years some pioneer work has been started in Osborne's laboratory at the Connecticut Agricultural Experiment Station in New Haven on the proteins of green plant tissue. Their extraction, isolation, and chemical characterization and analysis have been pursued by Osborne, Chibnall and their associates, and by Davies, at Cambridge, England, using much the same methods. The results of the Van Slyke analyses of such proteins and of the proteins of roots and other vegetable storage parts are summarized in Table 10. As would be expected from the variety of materials studied, the analytical results do not yield to general description. The high lysine content of many of these proteins conform with the general belief that roughages are excellent supplements to the grains in the feeding of farm animals.

The analytical results obtained with animal proteins, collected in Table 11, are of interest from two standpoints, *i.e.*, in assessing their value as foods, and in affording some light upon animal requirements for amino acids as these relate to the construction (growth) of animal tissues. The protoplasmic proteins are almost universally rich in lysine, containing the equivalent of 7 or more per cent of lysine nitrogen. Their histidine content is quite variable, even in muscle tissue, in part due possibly to a variable retention of blood, the hemoglobin of which is relatively rich in histidine. However, even the isolated proteins of muscle exhibit a wide range in their content of histidine. It is noteworthy that the arginine content of animal proteins is relatively moderate, and rarely attains the size shown by many vegetable proteins. The peculiar amino acid deficiencies of gelatin, the cystine poverty of casein but not of lactalbumin, and the apparent absence of cystine from hemoglobin are worthy of mention.

Within recent years a large amount of chemical data has accumu-

lated relating to the proteins of fish. Some of these data have been summarized and discussed by Tressler in the appendix to the report of the U. S. Commissioner of Fisheries for 1925 (published by the Department of Commerce as Bureau of Fisheries Document No. 1000). Other analyses of the proteins of other sea animals, obtained in the main by modifications of the esterification method of Fischer, will be found in another article by Okuda and associates cited in reference No. 103 in the bibliography to the tables of this section. A number of results of Van Slyke analyses of fish muscle proteins by Sekine and Akiyama (cited by Kimura) are included in Table 11. They reveal a remarkable difference between male and female fishes with reference to the amino acid content of their muscle proteins. The females show a slightly lower content of arginine and lysine nitrogen, but a distinctly higher content of histidine nitrogen. These results confirm earlier results by Sekine (104, bibliography to Tables) and this interesting sex difference is in turn confirmed by Kimura's results on warm blooded animals cited in Table 11. The histidine difference is particularly striking in these data of Kimura on male and female rabbits, oxen and chickens.

The Amino Acid Content of the Mixed Proteins of Feeds

In practical dietetics and in the feeding of farm animals, isolated proteins are, of course, not fed as such, but rather the mixtures of proteins contained in available foods and feeding materials. It has, therefore, occurred to some investigators to apply to such materials, either as such or after removal of non-protein nitrogenous material and to some extent of carbohydrates, the Van Slyke method of protein analysis so widely used with pure protein preparations. The difficulties and uncertainties involved in such an application of the method have been fully realized and discussed in the articles reporting the results secured. It has been shown that the non-protein nitrogenous constituents of feeds may interfere with the Van Slyke method, and in particular with the analysis of the bases, since this analysis is based on the assumption that phosphotungstic acid under the prescribed conditions will precipitate only arginine, histidine, lysine and cystine. It has also been shown that the presence of carbohydrates may occasion the decomposition of certain of the amino acids during the acid hydrolysis, resulting in excessive humin formation.

In spite of these faults, the results obtained are worth citing, since they appear to be, in the main, consistent with the results obtained by the same method on proteins isolated from the feeds studied. The later results of Hamilton, Grindley and their associates are particularly

TABLE 12. *The Determination of the Basic Amino Acids of Certain Feeds and Food Materials According to the Method of Van Slyke.*

Food Material	Percentage Total Nitrogen in Form of: Arginine	Histidine	Lysine	Cystine	Non-proteins	Reference No.*
Seeds						
Corn	8.7	4.8	2.2	1.1	9.8	95
Corn	7.7	2.5	2.1	1.6	...	96
Oats	11.6	5.8	2.8	0.9	12.9	95
Wheat	8.0	1.7	2.5	1.3	...	98
Wheat	9.0	1.7	2.6	0.9	...	96
Barley	9.5	3.6	2.2	1.3	...	98
Rye	10.5	10.5	1.2	2.2	...	97
Kafir corn	7.2	1.3	1.7	1.0	...	96
Cottonseed meal	18.7	7.2	4.2	0.9	6.2	95
Cottonseed flour	19.7	3.9	6.7	1.1	...	96
Linseed meal	15.9	6.1	3.7	1.1	8.4	99
Hemp seed	21.4	3.0	6.7	2.0	...	97
Sunflower seed	16.8	4.6	4.9	3.0	...	97
Tomato seed	16.3	0.0	8.4	1.3	...	96
Soybean	15.7	5.6	6.2	1.5	6.3	99
Soybean	15.5	2.6	7.0	1.5	...	97
Cow pea	17.7	3.6	6.0	1.2	...	96
Jack bean	9.8	6.1	9.9	0.8	...	96
Peanut	20.8	6.1	5.3	0.8	...	97
Black walnut	23.8	6.0	3.5	1.3	...	97
Hickory nut	24.2	6.7	3.4	1.6	...	97
Mill and Factory By-products						
Wheat bran	12.0	7.3	3.9	0.8	16.5	99
Wheat gluten	7.6	5.6	0.5	1.9	...	97
Gluten flour	8.9	5.2	0.4	2.1	...	97
Corn germ	11.0	5.8	5.6	0.0	...	96
Distiller's dried grains	11.3	0.0	4.8	3.0	...	97
Tankage	14.1	4.9	7.5	1.3	...	98
Tankage	12.3	2.2	2.5	2.5	...	97
Blood meal	9.2	8.5	9.7	0.7	...	98
Dried blood	7.7	8.4	10.0	2.0	...	97
Skim milk	8.7	4.9	8.2	1.2	...	98
Roughages						
Alfalfa hay	8.0	3.9	4.4	1.0	19.1	95
Red clover hay	6.9	5.0	2.6	0.9	13.5	99

* List of references will be found on pages 192 to 194.

valuable, since they are not complicated by the presence of non-protein nitrogenous substances or of any considerable amounts of carbohydrates.

The results obtained on the mixed proteins of feeds, in so far as they appear to be reliable, or at least in so far as they are not obviously unreliable, are summarized in Table 12. Many of Nollau's results have been omitted because of obviously faulty analyses for lysine and cystine,

TABLE 13. *The Amino Acid Content of a Number of Proteins, Determined Mainly by Isolation Methods.**

Amino Acid	Gelatin	Casein	Lactalbumin	Egg Albumin	Gliadin	Zein	Edestin
Glycine	25.5[71]	0.4[73]	0.4[75]	0.0[76]	0.0[77]	0.0[79]	3.8[82]
Alanine	8.7[71]	1.8[73]	2.4[75]	2.2[76]	2.0[78]	9.8[79]	3.6[82]
Valine	0.0[71]	7.9[73]	3.3[75]	2.5[76]	3.3[77]	1.9[79]	+[84]
Leucine-isoleucine	7.1[71]	9.7[73]	14.0[75]	10.7[76]	6.6[77]	25.0[80]	20.9[82]
Aspartic acid ..	3.4[71]	4.1[74]	9.3[75]	6.2[106]	0.8[106]	1.8[80]	10.2[106]
Glutamic acid..	5.8[71]	21.8[73]	12.9[75]	13.3[106]	43.7[77]	31.3[80]	19.2[106]
Hydroxyglutamic acid ..	0.0[71]	10.5[74]	10.0[75]	...	2.4[105]	2.5[80]	...
Serine	0.4[71]	0.5[73]	1.8[75]	...	0.1[78]	1.0[79]	0.3[82]
Proline	9.5[71]	8.0[74]	3.8[75]	3.6[76]	13.2[77]	9.0[79]	4.1[83]
Hydroxyproline	14.1[71]	0.2[73]	...	...	...	0.0[80]	2.0[82]
Phenylalanine..	1.4[71]	3.9[73]	1.2[75]	5.1[76]	2.3[78]	7.6[80]	3.1[83]
Tyrosine	0.01[71]	6.5[8]	1.9[75]	4.0[8]	3.1[8]	5.9[8]	4.5[8]
Cystine	0.17[46]	0.3[10]	4.0[10]	0.9[10]	2.4[46]	0.8[10]	1.0[10]
Arginine	9.1[72]	5.2[107]	3.0[2]	6.0[107]	3.2[78]	1.8[81]	15.8[85]
Histidine	0.9[71]	2.6[47]	1.5[2]	2.3[47]	2.1[47]	1.2[47]	2.1[85]
Lysine	5.9[71]	7.6[73]	8.4[2]	3.8[76]	0.6[2]	0.0[79]	2.2[85]
Tryptophane ..	0.0	2.2[10]	2.7[10]	1.3[8]	0.8[8]	0.17[8]	1.5[8]
Ammonia	0.4[71]	1.6[73]	1.3[75]	1.3[76]	5.2[77]	3.6[79]	2.3[39]
Total.....	92.4[a]	94.8	81.9	63.2	91.8	103.4	96.6[a]

* List of references indicated will be found on pages 192 to 194.

[a] According to S. B. Schryver and H. W. Buston (*Proc. Roy. Soc., London,* 1925, Series B, xcviii, 58), "gold label" gelatin contains 0.3 per cent of hydroxylysine, and edestin from hemp seed 3.3 per cent. No trace of this amino acid was found in either casein or egg albumin.

and, in the case of the pecan nut, of histidine. The accuracy of those results of Nollau included in the table cannot be assessed highly because of the obvious inaccuracies in so much of his work.

Obviously the practicability of these results is limited by the fact that they relate to the feed itself, rather than to its digestible fraction, which may or may not be representative of the whole.

THE SUMMATION OF THE AMINO ACIDS FOUND IN CERTAIN PROTEINS OF GENERAL INTEREST

The extent to which the analysis of proteins into their constituent amino acids accounts for their complete constitution is always a matter of interest to chemist and biologist alike, and it is therefore a matter of importance to balance the accounts at times in order to see how near the ultimate goal is. This has been done in Table 13 for a number of proteins, upon which the most work has been done or the most interest is centered. Most of the values there given have been obtained by gravimetric methods, but the results given for tyrosine, cystine and tryptophane and a few of those for histidine represent what appear to the authors to be the most reliable results of recently devised colorimetric methods.

TABLE 14. *Recent Determinations of the Amino Acid Content of Proteins, Mainly by Isolation Methods.**

Amino Acid	Arachin	Coconut Globulin	Kafirin	Stizolobin	Soluble Protein of Swede Turnips	Silk Fibroin
Glycine	0.0[86]	trace[87]	0.0[88]	1.7[89]	0.3[90]	40.5[91]
Alanine	4.1[86]	4.1[87]	8.1[88]	2.4[89]	3.6[90]	25.0[91]
Valine	1.1[86]	3.6[87]	4.3[88]	2.9[89]	9.9[90]	...
Leucine-isoleucine	3.9[86]	6.0[87]	15.4[88]	9.0[89]	9.0[90]	2.5[91]
Aspartic acid	5.6[106]	5.1[87]	2.3[88]	9.2[89]	7.0[90]	...
Glutamic acid	19.6[106]	19.1[87]	21.2[88]	14.6[89]	3.2[90]	...
Hydroxyglutamic acid	...	0.0[87]	...	2.8[89]	...	...
Serine	...	1.8[87]	...	0.7[89]	...	1.8[91]
Proline	1.4[86]	5.5[87]	7.8[88]	4.0[89]	4.2[90]	1.0[91]
Hydroxyproline	...	...	...	...	...	...
Phenylalanine	2.6[86]	2.0[87]	2.3[88]	3.1[89]	4.5[90]	1.5[91]
Tyrosine	5.5[86]	3.2[87]	5.5[88]	6.2[89]	2.9[90]	11.0[91]
Cystine	1.1[10]	1.5[10]	0.8[88]	1.5[10]	...	...
Arginine	12.5[86]	15.9[87]	1.6[88]	7.1[89]	3.1[90]	...
Histidine	2.1[86]	2.4[87]	1.1[88]	2.3[89]	3.0[90]	...
Lysine	1.7[86]	5.8[87]	0.9[88]	8.5[89]	4.3[90]	...
Tryptophane	0.9[10]	1.2[10]	0.9[10]	1.4[10]	...	...
Ammonia	2.0[86]	1.6[87]	3.5[88]	1.5[89]	1.2[90]	...
Total	64.1	78.8	75.7	78.9	56.2	83.3

* List of references indicated will be found on pages 192 to 194.

It is evident that for several proteins over 90 per cent of the molecule has been accounted for, not allowing for hydrolytic water. The more than perfect summation for zein may be accounted for by the water added during hydrolysis.

Some recent fairly complete analyses of other proteins are given in Table 14. For coconut globulin, kafirin, and stizolobin, the results for arginine, histidine and lysine were obtained by the Van Slyke method, since no gravimetric results are available.

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BIBLIOGRAPHY ON THE PREPARATION OF AMINO ACIDS

Directions for the preparation of amino acids, either by isolation from protein hydrolysates or by synthesis, are of great aid to those investigating the physiological effects and the metabolic fate of these important substances. However, this monograph is concerned with principles rather than with technical details. The principles involved in methods that have proven valuable in the preparation of amino acids

from protein material are to a large extent the same as the principles upon which methods for their analytical determinations in proteins have been based. Since these principles have been discussed in preceding sections, it is superfluous to re-discuss them here.

A list of literature citations to methods that have proven useful in the preparation of amino acids is appended to this chapter in the hope that it will prove helpful. In compiling this list no attempt has been made to make it exhaustive, except in the case of the most recent developments. Some of the earlier references are cited, especially if the methods described have not been improved upon by later methods or if there are no later methods. Many references have been included because of their bibliographies alone. Chief among the earlier methods is the Fischer esterification procedure, by the use of which most of our present knowledge concerning the physical and chemical properties of the various amino acids have been obtained. This method is still the chief means of preparing many of the amino acids, particularly the monoamino monocarboxylic acids.

At the outset, reference may be made to the various editions of Abderhalden's Biochemisches Handlexikon, appearing in 1911, 1915 and 1924, and to the Handbuch der biologischen Arbeitsmethoden, also edited by Abderhalden. In these volumes, under the headings of the various amino acids, will be found a more or less complete list of references on the preparation of each amino acid.

General methods for the synthesis of the amino acids will be found in the following references: Erlenmeyer, E., Jr., *Ann. Chem.*, 1893, cclxxv, 1; Barger, G., and Ewins, A. J., *Biochem. J.*, 1917, xi, 58; and Harington, C. R., and McCartney, W., *Biochem. J.*, 1927, xxi, 852.

References to special methods for the preparation of the amino acids are listed below, under the names of the individual acids, which have been arranged alphabetically.

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CHAPTER IV

THE LIBERATION OF THE AMINO ACIDS IN THE INTESTINAL TRACT

While traces of amino acids and their simpler conjugation products are present in many food materials, and while a few foods, particularly roots, tubers, and fresh green vegetation, may contain considerable amounts, in practical nutrition the main sources of amino acids to animals are the proteins contained in the food consumed. A discussion of the agents responsible for the liberation of the amino acids from these complexes, of the processes of liberation, and of the completeness of such processes, seems a necessary preliminary to any consideration of the fate of the amino acids and of their functions in animal metabolism.

THE GENERAL FEATURES OF PROTEOLYSIS AND PROTEASES

The liberation of amino acids from dietary protein occurs within the intestinal tract, and is an important phase of digestion. The processes of digestion are concerned not only with the reduction of the food nutrients to a soluble and diffusible form, in which they can readily pass through the membranes lining the intestines into the blood and lymph, but they are also concerned with the production of substances upon which the cells of the body can subsist. As a matter of fact, the first mentioned function of digestion may be completed before the second, in the sense that soluble and diffusible products may be produced that cannot serve as food to the cells without still further disintegration. Maltose is a soluble and diffusible product of the digestion of starch, but it cannot serve as food to the cells until it is split into two molecules of glucose. Similarly, peptides are soluble and diffusible products of the digestion of proteins, but until they are broken down to their constituent amino acids, they apparently can serve no useful function in the body. These final cleavages of absorbable products of digestion will normally occur within the intestinal tract, or within the mucosa lining it. However, should these soluble but incompletely digested products gain access to the circulating fluids, the cells themselves, to a limited extent, possess the capacity of completing their digestion.

The chemical processes brought about by the agents secreted by the

digestive glands are entirely hydrolytic cleavages, *i.e.*, cleavages associated with the addition of the elements of water at the point of cleavage. They involve but little loss of chemical energy, so that the end products of digestion contain very nearly as much energy as the nutrients from which they originated, a fact of no inconsiderable importance.

The agents bringing about the hydrolytic cleavage of the nutrients in digestion are called enzymes; they are secreted by the glands lining the different segments of the alimentary canal or connected with the canal by ducts, through which their secretions are poured. The digestive enzymes are ordinarily considered to be catalysts in the sense that they do not enter into the composition of the products of their action, to a measurable extent at least, and hence can convert an unlimited amount of substrate (the food nutrient upon which it acts) into its respective products of complete hydrolysis, except in so far as the enzyme is itself destroyed by secondary reactions. For this reason, a given amount of enzyme may digest many times its weight of substrate. Thus, it has been found that 1 part of pepsin will dissolve *in vitro* approximately 3000 parts of egg albumin in the course of 3 hours; *in vivo,* its activity is probably even more extensive.

Enzymes are specific in their action to a greater or less extent. The action of any enzyme is confined to some particular class of nutrients, and in some cases to some individual substance. However, the proteases are specific only in the sense that their action is confined to proteins. The different proteases may be distinguished from each other by the hydrogen ion concentrations most suitable to their activity, by the linkages within the protein molecule that they most readily attack, or by their inability to disintegrate certain proteins or protein derivatives.

The differential activity of digestive proteases in hydrolyzing proteins may be most readily explained on the assumption that there are different types of linkages between amino acids within the protein molecule. This explanation has been advanced by Abderhalden,[1] who believes that besides the well-known acid amide union of amino acids to form peptides, there may be ester unions involving the hydroxyl groups of the hydroxy-amino acids (serine, tyrosine, hydroxy-proline, hydroxy-tryptophane, and hydroxy-glutamic acid), diketopiperazine ring structures, and anhydride unions. In support of the belief that anhydride unions exist in the protein molecule between adjacent amino acids, Abderhalden has isolated from the acid hydrolysate of goose

[1] Abderhalden, Emil: Über die Struktur der Proteine. *Z. physiol. Chem.*, 1923, cxxviii, 119. Also by the same author, Weitere Studien über den stufenweisen Abbau von Eiweissstoffen. *Z. physiol. Chem.*, 1923, cxxix, 106.

feathers a product containing one molecule of hydroxyproline, two molecules of proline, and one molecule of glycocoll in anhydride union. He also cites the finding by Levene and Wallace [2] of *l*-prolyl-glycinanhydride in the digestion of gelatin, and the finding by Dakin [3] of *d*-isoleucyl-*d*-valinanhydride in casein hydrolysates and of *γ*-hydroxyprolyl-proline anhydride in gelatin hydrolysates. It is reasonable to suppose that these different linkages are broken with different degrees of facility by proteases and that one protease may attack a given linkage much more readily than another protease.

The hydrolysis of a protein by enzymes or by inorganic catalysts is a process of erosion, consisting of cleavages into smaller and smaller fragments, with a simultaneous release of individual amino acids. As this hydrolytic erosion proceeds the hydrolysate loses successively the chemical and physical properties characteristic of proteins. The property of precipitability by alkaloidal reagents, or by salts of the heavy metals, or by mineral acids, or by alcohol, or the property of being "salted out" of solution, are lost by the protein hydrolysate at different stages of hydrolysis. The intermediate products of this hydrolysis are, in fact, differentiated from each other by the characteristic protein precipitation reactions still retained by them. Since most of the color reactions of protein depend on the presence of certain amino acids in the molecule, they are not affected in any way by the stage of hydrolysis. However, the biuret color test varies with the stage of hydrolysis, and is finally negative even before hydrolysis may be assumed to be complete, because it depends upon the existence of a limited number of types of linkages between amino acids.

METHOD OF MEASURING THE RATE AND EXTENT OF PROTEOLYSIS

From a chemical standpoint, the most significant phenomenon in a hydrolyzing protein digest is the liberation of those chemical groups characteristic of the amino acids, *i.e.*, carboxyl and *α*-amino groups. Obviously, for each acid amide group hydrolyzed, one each of these groups would be liberated. Therefore, two methods are available for following quantitatively the course of such hydrolyses, one concerned with the determination of the NH_2 groups liberated, and the other concerned with the determination of the COOH groups liberated. The Van Slyke gasometric method [4] is admirably adapted to the determination of *α*-amino nitrogen, making use of the nitrous acid reaction,

[2] Levene, P. A., and Wallace, G. B., *Z. physiol. Chem.*, 1906, xlvii, 143. Also, Levene and Beatty, *Ber.*, 1906, xxxix, 2060.
[3] Dakin, H. D., *Biochem. J.*, 1918, xii, 290; *J. Biol. Chem.*, 1920, xliv, 524.
[4] Van Slyke, D. D., *J. Biol. Chem.*, 1913-14, xvi, 121.

while the Sörensen formol titration method [5] permits an accurate titration of the acidity of α-amino acids subsequent to the methylation of the amino groups by the use of formaldehyde.

During the hydrolysis of protein by proteases, the electrical conductivity of the hydrolysate will continuously increase, due to the liberation of ionizable radicals.[6] This increase in conductivity may therefore be used in studying the rate of proteolysis *in vitro*.[7] If it is desired to follow the rate of change in the original protein, independent of the simultaneous changes in the intermediate products of hydrolysis, the hydrolysate may be tested by reactions relating to the protein alone; thus, the decrease in nitrogenous material coagulated by heat, or precipitated by protein reagents, or salted out of solution, may be studied. The decrease in viscosity of the solution has also been used for the same purpose.[8]

It must be obvious that no two of the methods mentioned will give equivalent results, and it would be a rash undertaking to select the method that measures the most accurately the "true" rate of digestion, if such an ideal can be assumed to exist in a solution in which so many different reactions are proceeding simultaneously. However, there seems to be a general consensus of opinion that the liberation of α-amino nitrogen is a fairer measure of the rate of digestion than any other, and that the completeness of digestion at any period of time is best expressed by the free amino nitrogen in per cent of the total amino nitrogen as determined by prolonged acid hydrolysis.

THE THEORY OF ENZYMATIC PROTEOLYSIS

It is not within the province of this discussion to consider at any length the theory of enzyme action. However, a brief notice of notable contemporary work on the so-called catalytic action of proteases on proteins will aid materially in completing the picture of protein digestion in the alimentary tract.

The kinetics of enzyme action has been the subject of a large number of experimental and theoretical studies. The results of such studies, particularly with proteases, have almost invariably shown that enzyme action does not follow the general laws of chemistry, in particular the law of mass action. It would be expected from theoretical considerations that the action of an enzyme on its substrate should conform to the equation expressing the velocity of a monomolecular reaction,

[5] Sörensen, S. P. L., *Biochem. Z.*, 1908, vii, 45.
[6] Sjöqvist, J., *Skand. Arch. Physiol.*, 1893-95, v, 277.
[7] Northrop, J. H., *J. Gen. Physiol.*, 1919, ii, 113; 1920, iii, 211; and other papers.
[8] Northrop, J. H., *J. Gen. Physiol.*, 1923, v, 353.

accelerated by a catalyst, but such is not the case, except in a few instances.

The activity of proteases in particular has been difficult to reconcile with the laws of homogeneous solutions. The rate of formation of the products of proteolysis does not, for example, increase in proportion to the concentration of protein, but much more slowly, eventually being nearly independent of it. Also, increasing concentrations of protease do not proportionately increase the rate of proteolysis. These experimental facts have led Bayliss [9] and others to the conclusion that enzyme reactions are not homogeneous and cannot be reconciled with the laws of homogeneous solutions. The known colloidal nature of enzymes has suggested that the concentration of some adsorption complex of substrate and enzyme determines the rate of reaction, rather than the concentrations of substrate and enzyme separately.

Other investigators have assumed that the concentration of an enzyme-substrate complex determines the rate of enzyme reactions, without, however, introducing the adsorption concept. On such a basis, for example, Brown [10] has suggested that the relative decrease in the rate of digestion with increasing substrate concentrations is due to the fact that the enzyme becomes saturated with substrate as the ratio of substrate to enzyme concentration increases. Van Slyke and Cullen [11] among others have derived an equation based on such a mechanism expressing the rate of enzyme action.

However, there are objections to each of these two general methods of explaining enzyme action. While it may be possible to explain the kinetics of enzyme action on the basis of several different sets of assumptions, and to derive mathematical formulas that fit experimental data satisfactorily, the mere existence of a good agreement between calculated and observed values, particularly if the mathematical equations involve several constants to be determined from the data themselves, cannot be considered conclusive evidence of the correctness of the assumptions originally made. Direct experimental verification of these assumptions is essential. For example, the assumption that the rate of enzyme action depends upon the concentration of a substrate-enzyme combination rather than on the concentration of the reacting substances cannot be considered as proven because a mathematical formula based on this assumption permits the successful prediction of experimental results. It must, itself, be subjected to direct experimental inquiry. In

[9] Bayliss, W. M., "The Nature of Enzyme Action," New York, 3rd edition, 1914. Also Armstrong, E. F., and Armstrong, H. F., *Proc. Roy. Soc. London,* Series B, 1913, lxxxvi, 561.
[10] Brown, A. J., *J. Chem. Soc.,* 1902, lxxxi, 373.
[11] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.,* 1914, xix, 141.

fact, as Northrop has pointed out,[12] "the term in the various equations which have been derived for enzyme hydrolysis and which has been supposed to represent an equilibrium between the substrate and enzyme could be interpreted just as well as representing an equilibrium between the substrate and water. The only difference would be as regards the physical significance of the constants."

The fact that proteins and proteases are colloidal in nature does not preclude the possibility of explaining their interaction by general physico-chemical laws. There is a growing tendency to deal with the behavior of protein solutions according to such laws, and considerable success has already been realized. Thus, Chick and Martin [13] have demonstrated that the heat coagulation of proteins follows the course of a monomolecular reaction when the hydrogen ion concentration is kept constant, while Bosworth and Van Slyke [14] have shown that casein and paracasein form definite salts with calcium and other bases. In the same connection, Svedberg [15] has shown that colloidal solutions obey the gas laws much better, in fact, than do ionized substances, while only recently Sörensen [16] has shown that the conceptions developed from a study of real solutions may be applied successfully to a study of protein solutions, in particular, and has stated his belief that only by the application of purely chemical and physico-chemical theories is it possible to make appreciable headway in the solution of problems relating to the serum globulins. There is every reason to believe, therefore, that reactions in colloidal solutions should obey the law of mass action, and in particular that enzyme reactions may be thus simply explained.

NORTHROP'S WORK

Northrop has, in fact, succeeded in explaining the action of pepsin and trypsin entirely in chemical terms, and in checking up experimentally each item in his explanation, so that his conclusions are peculiarly convincing, even to the non-mathematical student. He has pointed out that the law of mass action deals with the "active" concentrations of reacting substances, which may not coincide with their total concentrations. The distinction between active and total concentrations of reacting substances has already been drawn in explaining purely inorganic reactions and is based upon the ionization theory. In particular, the speed of the acid hydrolysis of proteins is assumed to be a func-

[12] Northrop, J. H., *J. Gen. Physiol.*, 1924, vi, 423.
[13] Chick, H., and Martin, C. W., *J. Physiol.*, 1910, xl, 403; 1911, xliii, 1; 1912, xlv, 61, 261
[14] Bosworth, A. W., and Van Slyke, L. L., *J. Biol. Chem.*, 1913, xiv, 207, 211.
[15] Svedberg, T., *Z. physik, Chem.*, 1910, lxxiii, 547; Svedberg, T., and Inouye, K., *ibid.*, 1911, lxxvii, 145.
[16] Sörensen, S. P. L., *J. Amer. Chem. Soc.*, 1925, xlvii, 457.

tion not of the total concentration of acid, but of the concentration of hydrogen ion. In this case the assumption is capable of verification, since the concentration of hydrogen ions may be determined by several independent methods which give approximately the same results, all verifying the assumption. If this were not the case, the kinetics of acid hydrolysis would be as difficult to interpret as the kinetics of enzyme hydrolysis. The active concentration of substrate and enzyme in their interaction cannot be so directly and convincingly determined as the active concentration of an acid catalyst, and, to complicate the problem further, it may be subject to more than one equilibrium.

The active concentration of the digestive proteases, pepsin and trypsin, is modified by two independent reactions, one a spontaneous and irreversible inactivation, increasing in velocity with rising temperature, the other a reversible inactivation of the enzyme due to an inhibiting influence of some of the products of its action on the substrate. Northrop was able to show, by experiments designed to accentuate the reaction studied and to minimize other interfering reactions, that for both pepsin [17] and trypsin,[18] the inactivation of the enzyme proceeds according to the law of mass action. The inhibiting products of the reaction are not amino acids, but for both enzymes are intermediate hydrolytic products, possibly peptones in the case of pepsin, and peptides in the case of trypsin. It is probable that a dissociable compound is formed between enzyme and inhibiting substance, and that the compound (enzyme + inhibitor) is inactive proteolytically. The inactivation of the two proteases may be accurately represented mathematically by equations based upon this assumption, and the probability of the existence of such an equilibrium is greatly enhanced by the observation [19] that the inhibiting substance or substances protects the enzyme against spontaneous inactivation, *i.e.*, the compound is stable as well as inactive.

Northrop's experimental work has led him to believe that the rate of hydrolysis of proteins by both pepsin and trypsin is proportional to the concentration of *uncombined* enzyme, and he has been able very successfully to explain all of his observations on this basis by means of the law of mass action. This view is diametrically opposed to the theory, quite generally accepted in explaining enzyme action, that the rate of hydrolysis is proportional to the concentration of some compound between enzyme and substrate, the subsequent decomposition of which liberates the free enzyme and the products of the reaction. While

[17] Northrop, J. H., *J. Gen. Physiol.*, 1920, ii, 471.
[18] Northrop, J. H., *J. Gen. Physiol.*, 1920, iii, 211; 1922, iv, 245, 251; 1922-23, v, 335, 751.
[19] Northrop, J. H., *J. Gen. Physiol.*, 1922, iv, 261.

it is quite probable that such a compound is formed in proteolysis, it is quite improbable that any appreciable amount of pepsin or trypsin is combined with the substrate at any one time, and that under any conditions the protease becomes "saturated" with substrate.

The following facts are cited by Northrop as militating against the belief that the kinetics of peptic and tryptic proteolysis is determined by the amount of enzyme combined with the substrate rather than by the amount of free enzyme.

1. While the intermediate products of hydrolysis protect the enzyme against spontaneous inactivation, the substrate exerts no protection whatever: the enzyme becomes inactivated at the same rate in the presence of its substrate as in "pure" solution.[20]

2. The relative velocity of hydrolysis of any two substrate concentrations is independent of the enzyme concentration used to make the comparison.[21] This is contradictory to the assumption that the velocity is dependent upon the concentration of a compound between enzyme and substrate, since if this were true, a greater amount of substrate would be required to saturate a greater amount of enzyme.

3. The equilibrium between enzyme and the inhibiting products of its action is unaffected by the concentration of substrate. However, if the substrate were also combined with the enzyme, an increase in substrate concentration should affect the inactivation of the enzyme by the products of its action.[22]

Therefore, the observed facts in Northrop's investigations cannot be accounted for on the basis of the formation of a compound between enzyme and substrate, if it be assumed that this compound is governed by the law of mass action. The assumption of the existence of such a compound was originally advanced in explanation of the phenomenon, common to all enzyme reactions, that the rate of formation of the products of the reaction does not increase in proportion to the concentration of substrate, but much more slowly, eventually being nearly independent of it. However, it seems necessary to conclude from Northrop's experiments that the relative decrease in the rate of digestion of protein solutions of increasing concentration is independent of the enzyme concentration used. It would, therefore, seem to be due to some equilibrium in the protein solution itself, rather than to an equilibrium between protein and enzyme.

The equilibrium that first suggests itself is a dissociation equilibrium,

[20] Northrop, J. H., *J. Gen. Physiol.*, 1921-22, iv, 266; Ringer, W. E., *Z. physiol. Chem.*, 1921, cvi, 107.
[21] Northrop, J. H., *J. Gen. Physiol.*, 1919-20, ii, 595.
[22] Northrop, J. H., *J. Gen. Physiol.*, 1922, iv, 487.

defining the relative concentration of unionized and ionized protein. It is well known that in acid solution protein exists in an ionized condition. Furthermore, the concentration of ionized protein would not be directly proportional to the total concentration of protein, but would increase more slowly than the total concentration. It is obvious *a priori,* therefore, that the rate of digestion of protein by pepsin in acid solution will be more nearly proportional to the concentration of ionized protein than to the concentration of total protein; the hypothesis that it is the ionized protein only which takes part in the reaction will permit a closer approximation to the observed rate of reaction. Pauli [23] was the first to suggest this hypothesis, though he presented no direct evidence in support of it. Northrop [21] tested the hypothesis experimentally, and was able to show that the rate of digestion by pepsin of protein solutions of varying concentration was proportional to their ionization at the same pH, as measured by their conductivity. He was thus able to predict quite accurately the rate of hydrolysis of a given protein solution from a measurement of the concentration of ionized proteins. Furthermore, the pH corresponding to minimum digestion shifted with the isoelectric point of the protein.

These observations were later confirmed and extended to include a similar study of tryptic proteolysis.[24] The results of these later experiments demonstrated that the rate of digestion of gelatin, casein, and hemoglobin by pepsin or trypsin may be predicted from the amount of protein ion present. The acid salt of these proteins is rapidly attacked by pepsin, and the alkali salt by trypsin. The curve for the rate of digestion, plotted as a function of the pH, is therefore nearly identical with the titration (dissociation) curve * of the proteins. The same result was found with edestin and globin, the alkali titration curve of these proteins agreeing with the rate of tryptic hydrolysis. The rate of digestion was found to be a minimum at the isoelectric point of the protein and a maximum at that pH at which the protein is completely combined with acid or alkali to form a salt.

Northrop's work on the proteases pepsin and trypsin necessitates a revision of several current ideas concerning enzymes and their action. In the first place, they can no longer be considered catalysts in the classical sense, if they combine in appreciable amounts with the products of their action and thus affect the final equilibrium attained. That such

[23] Pauli, W., *Arch. ges. Physiol.,* 1910, cxxxvi, 483.
[24] Northrop, J. H., *J. Gen. Physiol.,* 1922, v, 263.
* The titration curves were determined by adding a known amount of standard alkali or acid to the protein solution and determining the pH. A similar series was carried out in which the protein was omitted. The amount of protein combined with the acid (or alkali) was then determined by subtracting the volume of acid (or alkali) required to bring water to any given pH from the volume required to bring an equal volume of the protein solution to this pH.

combination does occur for pepsin and trypsin, Northrop has shown, while Bodenstein and Dietz [25] have also shown experimentally that this is true in certain cases. Northrop suggests, therefore, that enzyme reactions be considered as bimolecular reactions in which one of the products dissociates more or less completely with the liberation of active enzyme; if the dissociation is complete the result would be a monomolecular reaction and, if no dissociation whatever takes place, a bimolecular reaction. Most enzyme reactions are apparently intermediate.

Current ideas concerning the effect of the reaction of the medium upon enzyme action are also in need of revision. One of the most striking peculiarities of enzyme reactions is the marked effect of acidity or alkalinity, each enzyme working best within a rather small range of hydrogen-ion concentration. The general tendency has been to assume that the influence of the pH of the medium was exerted upon the enzyme, but Northrop has shown that for pepsin and trypsin the effect is partly upon the substrate, though the spontaneous inactivation of the enzyme is related to the hydrogen-ion concentration. Thus, pepsin [26] in solution is most stable at 38° C. at pH 5.0. Increasing the H-ion concentration above this value causes a slow increase in the rate of destruction of the enzyme, while decreasing it below pH 5.0 causes a very rapid increase in the rate of destruction of the enzyme. Trypsin is also most stable at pH 5.0 and is rapidly destroyed in strongly acid or alkaline solutions.[19] Except for its destructive action on the enzyme, however, the hydrogen-ion concentration influences the rate of proteolysis by pepsin and trypsin by the formation with the substrate of acid or basic salts, the concentration of which, in turn, determines the concentration of ionized protein. The optimum pH for peptic and tryptic hydrolysis thus depends upon the protein substrate used, and is not a characteristic of the enzyme itself. As Northrop points out, this explanation of the hydrolysis of proteins by pepsin and trypsin cannot be extended directly to enzymes in general, since for many enzymes the substrate is not known to exist in an ionized condition at all.

GASTRIC DIGESTION OF PROTEINS: PEPSIN

The gastric digestion of proteins consists mainly of the action of gastric protease, more commonly referred to as pepsin. Pepsin is responsible for a slow and not particularly extensive hydrolysis of proteins, characterized by the fact that free amino acids are not liberated, though biuret products may be separated from peptic digests. Accord-

[25] Bodenstein, M., and Dietz, *Z. Electrochem.*, 1906, xii, 605.
[26] Northrop, J. H., *J. Gen. Physiol.*, 1920, ii, 465.

ing to Abderhalden such products consist mainly of combinations of glycine, phenylalanine, and proline. Undoubtedly the main products of peptic hydrolysis are proteoses and peptones. There is considerable difference of opinion among investigators as to which of these two classes of intermediate hydrolytic products is the most important end product of peptic digestion. London is inclined to believe that proteoses form by far the largest part of the products of peptic action, while Abderhalden considers peptones to be the normal products of the action of this enzyme. Such divergent views, based in each case upon experimental evidence, can only be reconciled on the supposition that the end products actually resulting *in vivo* depend upon the rate of emptying of the stomach, and probably also upon the kind of protein. In any case, the question is not a matter of moment, since gastric digestion of proteins is merely a preliminary to the much more vigorous intestinal digestion. However, Abderhalden has shown that for the difficultly hydrolyzable proteins, such as elastin and other connective tissue proteins, peptic digestion may continue for some time in the small intestine, due to the fact that the enzyme is readily adsorbed by such proteins and carried through the pylorus with them.

THE DIGESTION OF CASEIN: RENNIN

The gastric digestion of casein, the main protein of milk, has excited a large number of investigations, because of the fact that gastric juice, particularly of young suckling mammals, exerts a marked coagulating effect on milk. The commercial importance of this reaction in the manufacture of cheese has also undoubtedly been a contributing factor of no small importance to the great interest taken in it. From the published reports of investigations dealing with the coagulation of casein by gastric juice, a number of discordant findings and inconsistent explanations are evident, relating particularly to the question whether there is in gastric juice an enzyme specifically concerned with the coagulation of casein, or whether this coagulation is simply an incident in the digestion of casein by proteases in gastric juice. Another point of difference relates to the nature of the products formed, and hence to the nature of the reaction itself. It would be entirely beyond the scope of this monograph to discuss in detail the controversies concerning this reaction. It must suffice to present what, in the opinion of the authors, seems to be the most reliable information and the most plausible interpretation of it.

The coagulation of milk by gastric juice is an enzyme action. The enzyme concerned in this action has been called rennin or, less fre-

quently in English and American writings, chymosin. However, any proteolytic enzyme, apparently, can bring about the same change in the same way, even plant proteases, which are, in fact, used in certain localities in cheese making. The question of the identity of rennin (chymosin) with pepsin has been answered both in the negative and the affirmative by different investigators. A reconciliation of these divergent views has been proposed recently by Hammarsten,[27] who has himself been identified with the theory that rennin is distinct from pepsin. Hammarsten points out that the dualistic view is based mainly on experiments with the stomach of the calf, while the unitarian view is based on investigations with the stomach of swine and dogs. He cites experimental evidence that in the stomach of the calf the rennin activity is much less stable in an acid medium than the pepsin activity, and much more stable in an alkaline medium, while in the pig's stomach the loss of rennin activity in either medium was approximately as rapid as the loss of pepsin activity. Furthermore, the rennin activity of stomach extracts from the calf rapidly decrease with increasing age of the animal, so that ultimately the stomach extracts of mature cattle have a rennin activity only one-sixtieth to one-twentieth of the stomach extracts of calves. Also, with the calf's stomach a separation of rennin and pepsin activity may be effected by dialysis of the extract, by precipitation with casein in the course of its coagulation, and by fractional extraction of the dried and pulverized mucosa by means of saturated NaCl solutions. Such separations have never been effected with the pig's stomach.

The work of Bosworth[28] on the nature of rennin action seems peculiarly convincing in showing that it consists of the cleavage of each molecule of casein into two molecules of a substance having the same composition as casein, but possessing one-half its molecular weight and one-half its acid valencies.[29] This compound is known as paracasein. Furthermore, the action is not *per se* a coagulation, since in the absence of calcium salts the paracasein does not precipitate. Its precipitation is a secondary effect. However, it is possible that the phenomenon of milk coagulation is in part a problem in colloid chemistry.[30]

INTESTINAL DIGESTION OF PROTEIN: TRYPSIN AND EREPSIN

The most vigorous protein hydrolysis occurs in the small intestine, due to the influence of two enzymes, trypsin and erepsin. The former

[27] Hammarsten, Olaf, *Z. physiol. Chem.*, 1923, cxxx, 55.
[28] Bosworth, A. W., *J. Biol. Chem.*, 1913, xv, 231; *ibid.*, 1914, xix, 397.
[29] Van Slyke L. L., and Bosworth, A. W., *J. Biol. Chem.*, 1913, xiv, 227.
[30] Bostrom, E. F., *Proc. Soc. Exp. Biol. Med.*, 1923-24, xxi, 301.

enzyme is secreted by the pancreas in the form of a proenzyme, trypsinogen, which is activated only when it reaches the small intestine and comes in contact with its activator, known as enterokinase. The trypsinogen-enterokinase mechanism evidently serves the purpose of protecting the secretory cells of the pancreas against the proteolytic activity of trypsin, since trypsin, though it normally acts in digestion only after peptic hydrolysis, nevertheless can vigorously hydrolyze native proteins. Pancreatic juice also contains erepsin in small concentration. While the activity of pancreatic erepsin is not ordinarily considered to be great, there is some evidence [31] indicating that it may be, especially if peptic proteolysis has been unusually complete.

Erepsin is secreted by the glandular cells lining the secreting tubules in the walls of the small intestine. Unlike trypsin it is apparently secreted in the active form, since it will not attack most native proteins. It is generally stated that casein and gelatin are the only proteins attacked by erepsin, though some doubt has been expressed as to the truth of this belief.[32] Although erepsin is contained in intestinal juice, most of the quantity of this enzyme elaborated in the intestinal wall is retained there, and very probably completes the last steps of protein digestion while the penultimate products of hydrolysis, the peptides, are being absorbed.

THE LIBERATION OF AMINO ACIDS

Tryptic hydrolysis of proteins is sharply distinguished from peptic hydrolysis by the rapid liberation of amino acids, indicating that the point of attack of trypsin is different from that of pepsin. Furthermore, different amino acids are liberated at different rates, either because some amino acids occupy a more exposed position in the protein molecule than others, or because certain amino acid linkages are more readily hydrolyzed than others. Tyrosine and cystine appear very soon in a tryptic hydrolysate. The following experimental results of Abderhalden [33] illustrate the more rapid liberation of tyrosine than of glutamic acid.

TABLE 15. *The Rate of Liberation of Tyrosine and Glutamic Acid in a Pancreatic Digest of Casein.*

(Results express percentage liberation of each amino acid.)

Days	1	3	6	9	11	13	17	21
Tyrosine	16.7	97.8	96.9	96.5	93.3	100.0	99.1	96.9
Glutamic acid	14.2	16.3	45.2	83.4	80.1	81.5	82.4	85.5

[31] Terroine, E. F., and Przylecki, S. J., *Arch. Internat. Physiol.*, 1923, xx 377.
[32] Frank-l. E. M., *J. Biol. Chem.*, 1916, xxvi, 54.
[33] Abderhalden, Emil, "Lehrbuch der Physiologischen Chemie," 5 Auflage, 1 Teil. Berlin, 1923, S. 482.

TABLE 16. *The Rate of Liberation of Tyrosine and Glutamic Acid in a Pancreatic Digest of Edestin.*

(Results express percentage liberation of each amino acid.)

Days	1	2	3	7	16
Tyrosine	78.4	97.6	97.6	100.0	100.0
Glutamic acid	4.3	7.4	10.9	31.1	60.2

In these digestion experiments other amino acids, *i.e.*, alanine, valine, leucine, aspartic acid, lysine, arginine, and histidine, were detected. Proline and phenylalanine, however, could not be detected except very occasionally. There thus appear to be peptide groupings in the protein molecule which are very resistant to the action of pancreatic juice, and which contain proline and phenylalanine. A proline fraction in casein comparatively resistant to tryptic proteolysis is also indicated by the work of Hunter.[34]

The presence of resistant amino acid groupings in proteins containing disproportionate shares of the several amino acids is also indicated by the *in vitro* digestion studies of Jones and Waterman [35] on arachin, the principal protein of the peanut. This protein was found to be relatively indigestible to pepsin, trypsin, and dilute alkali. From the alkali hydrolysate, a cleavage product amounting to about one-third of the original arachin was isolated and analyzed for basic amino acids by the Van Slyke procedure. This resistant fraction was found to contain about two-thirds of the total histidine, about one-third of the total arginine and of the total cystine, and about two-fifths of the total lysine of arachin. The similarity in the action of hot, dilute alkali, and of pepsin and trypsin on proteins, shown to exist by Northrop,[36] gives added biological significance to this finding.

Recently Hunter and Dauphinee [37] have reported a rapid cleavage of arginine from proteins during tryptic digestion. In the case of gelatin one-third of the total arginine was split off within 30 minutes, more than one-half in the course of 3 hours, and by the third day an equilibrium had been reached, at which almost exactly two-thirds of the total arginine was liberated. A similar rapid liberation of arginine was observed in the digestion of casein, and in this case also just one-third of the arginine linkages appeared to be resistant to trypsin. With edestin, however, the appearance of arginine was much more gradual: at the end of the sixth day only one-half of the total arginine was split off.

Fürth and Lieben [38] have reported experiments indicating that there

[34] Hunter, A., *Proc. Roy. Soc. Canada,* 1922, xvi, 71.
[35] Jones, D. B., and Waterman, H. C., *J. Biol. Chem.,* 1922, lii, 357.
[36] Northrop, J. H., *J. Gen. Physiol.,* 1921-22, iv, 57.
[37] Hunter, A., and Dauphinee, J. A., *Proc. Amer. Soc. Biol. Chem.,* December, 1924, p. 31.
[38] Fürth, O., and Lieben, F., *Biochem. Z.,* 1920, cix, 153.

is no ground for ascribing to tryptophane an especially exposed position in the protein molecule, since it is not for the most part set free in the first stages of tryptic hydrolysis, but parallels the cleavage of amino acids in general. After several weeks not more than one-third of the tryptophane was split off from fibrin by trypsin. However, Abderhalden claims that tryptophane, like tyrosine, is rapidly removed from proteins by tryptic action.

THE LIBERATION OF AMMONIA

Accompanying the liberation of amino acids during tryptic proteolysis is a liberation of ammonia, which, according to all indications, is present in the protein molecule in amide linkage. Since the quantity of ammonia liberated is closely related to the quantity of the dibasic amino acids in protein, it is presumed that one of the two carboxyl groups of these acids is involved in the amide union, and that this linkage accounts entirely for the ammonia liberated in proteolysis. Whether the liberation of ammonia from amides can be effected by proteases, or whether it requires a specific enzyme, a deamidase, is unknown. Hunter and Smith,[39] however, are inclined to the latter view. They compared the rate of liberation of ammonia with that of amino nitrogen in casein, gliadin, and Witte peptone subjected to the action of commercial trypsin. In all three cases, amide hydrolysis was found to take place very much less rapidly than peptide hydrolysis. The differences were so great as to suggest that the two processes are really effected by different enzymes, and that pure trypsin might produce no ammonia at all from proteins. Also, different preparations of trypsin seem to possess different relative rates of amide and peptide hydrolysis.

THE SYNTHETIC ACTION OF TRYPSIN

After long continued digestion of protein with trypsin, it has been found that a synthetic action leading to the production of amino acid anhydrides may be demonstrated. Thus, Fränkel and Feldsburg [40] have observed in the preparation of tryptophane by trypsin proteolysis that the bromine reaction of Nencki [41] for free tryptophane reaches a maximum intensity after several days, but that if the hydrolysis is allowed to continue for about 50 days with the addition of more trypsin, then the Nencki reaction disappears completely, although the Hopkins-Cole reaction, indicative of the presence of tryptophane in either the combined or free state, remains unaffected. The cause for this disappearance

[39] Hunter, A., and Smith, R. G., *J. Biol. Chem.*, 1925, lxii, 649.
[40] Fränkel, S., and Feldsburg, E., *Biochem. Z.*, 1921, cxx, 218.
[41] Nencki, M., *Ber. deutsch. chem. Ges.*, 1895, xxviii, 560.

of free tryptophane was found to be the conversion of *l*-tryptophane to *d*-tryptophane anhydride. The conversion of *l*-tyrosine into *d*-tyrosine anhydride was also demonstrated, though Fränkel and Jellinek [42] later showed that oxyproline did not undergo such a transformation. These investigations indicate that trypsin, or possibly an enzyme accompanying trypsin (called anhydrase), unites into an anhydride 2 molecules of the same amino acid through their carboxyl groups, with the cleavage of a molecule of water, the optical activity of the amino acid being reversed during the process.

Levene and Simms [43] have found that in the hydrolysis of peptides by either mineral acid or protease (erepsin) three reactions occurred simultaneously, according to the following scheme:

Anhydride $\rightleftharpoons$ Peptide $\longrightarrow$ 2 amino acid molecules.

The synthesis of peptide from amino acid was negligible. It was further shown that the resistance of different peptides to hydrolysis was approximately proportional to the product of the dissociation constants of the groups involved in the linkage. Similarly the stability of the anhydride bond was roughly proportional to the product of the constants of the groups involved.

Whether these simple physico-chemical considerations can explain all the marked differences, noted by Abderhalden and others, in the ease of hydrolysis among synthetic polypeptides cannot be decided at present.[44] It is worthy of passing comment that pepsin, of all the endocellular and extracellular proteases tested, has been found to be totally unable to hydrolyze synthetic peptides.

THE EXTENT OF PROTEOLYSIS IN THE DIGESTIVE TRACT

The extent to which the digestive proteases, severally or combined, can hydrolyze proteins has been the subject of a long series of investigations involving many different methods of measuring the rate and extent of hydrolysis. These *in vitro* experiments indicate that the combined action of pepsin, trypsin, and erepsin can effect a practically complete liberation of amino nitrogen.[45] Whether the small proportion of amino nitrogen not liberated by such procedures signifies an inability of the digestive proteases to cleave all the peptide complexes of protein, or whether it is merely the result of a piling up of end-products whose

[42] Fränkel, S., and Jellinek, P., *Biochem. Z.*, cxxx, 592.

[43] Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, 1925, lxii, 711. See also Levene, P. A., Simms, H. S., and Pfaltz, M. H., *ibid.*, 1924, lxi, 445; *also* Imai, T., *Z. physiol. Chem.*, 1924, cxxxvi, 192.

[44] For a good discussion of the enzymic hydrolysis of polypeptides, see the monograph of R. H. A. Plimmer, "The Chemical Constitution of the Proteins." Part 2, Synthesis. London. 1917.

[45] Dunn, M. S., and Lewis, H. B., *J. Biol. Chem.*, 1921, xlix, 343.

retarding action renders complete hydrolysis very difficult to attain and which *in vivo* would be removed by absorption, it is difficult to say. Considering the much more favorable conditions for hydrolysis existing within the alimentary canal than those existing in a laboratory receptacle, it is reasonable to assume that the complete breakdown of protein in the alimentary canal is possible, though under practical conditions of nutrition it may not be the rule. A striking illustration of the ease and rapidity of digestion *in vivo* is afforded by the results cited by Janney [46] on the rate of excretion of nitrogen in the urine following the ingestion of proteins and of amino acids. The rate of excretion of nitrogen in the urine appears to be the same whether amino acids are ingested as such or as protein; in other words, the rate of digestion is as great as or greater than the rate of metabolism of the amino acids. The alimentary canal can evidently digest protein completely in 9 to 12 hours, while in the laboratory many weeks are required.

Although there is little doubt but that, under the most favorable conditions, the alimentary tract possess the capacity to hydrolyze proteins completely, the evidence (or its interpretation) is to some extent contradictory concerning the activity of individual proteases. For example, the experiments of Frankel,[47] indicate that pepsin can liberate about 20 per cent of the total amino nitrogen of a protein, while trypsin can liberate about 50 per cent. However, trypsin acting upon protein partially hydrolyzed by pepsin effected a cleavage of about 70 per cent of the total amino nitrogen, and a comparison of the rates of tryptic proteolysis of native protein and of the same protein after a preliminary treatment with pepsin, indicates that they are much the same. The obvious conclusion is that pepsin attacks linkages in the protein molecule that cannot be split by trypsin, though the argument loses much of its force by reason of the fact that further additions of trypsin to a tryptic digest in which only 50 per cent of the amino nitrogen was liberated, increased the percentage liberation to 58 to 67. However, the conclusion of Frankel is in agreement with the observation of Fischer and Abderhalden [48] that neither proline nor phenylalanine were obtained from the tryptic digestion of casein, while both of these amino acids were recovered if a preliminary peptic digestion preceded the action of trypsin.

Northrop [49] has recently reinvestigated the question of the comparative hydrolysis of protein (gelatin) by pepsin and trypsin, and by acid

[46] Janney, N. W., *J. Biol. Chem.*, 1915, xxii, 191.
[47] Frankel, E. M., *J. Biol. Chem.*, 1916, xxvi, 31.
[48] Fischer, E., and Abderhalden, E., *Z. physiol. Chem.*, 1903-4, xl, 215.
[49] Northrop, J. H., *J. Gen. Physiol.*, 1921, iv, 57.

and alkali, using the formol titration to follow the liberation of carboxyl groups. His conclusions, which appear to be a fair interpretation of his data, are as follows:

1. Those linkages which are most rapidly split by pepsin or trypsin are among the more resistant to acid hydrolysis.
2. Those linkages which are hydrolyzed by pepsin are also hydrolyzed by trypsin.
3. Trypsin hydrolyzes linkages which are not attacked by pepsin.
4. Of the linkages which are hydrolyzed by both enzymes, those which are most rapidly hydrolyzed by pepsin are only slowly attacked by trypsin.
5. Those linkages which are attacked by trypsin or pepsin are among the ones first (most rapidly) hydrolyzed by alkali.

According to Northrop's data, therefore, the difference in tryptic proteolysis depending upon whether the protein has or has not been previously subjected to the action of pepsin, relates entirely to the rate of hydrolysis, not to the possible extent of hydrolysis. It appears, furthermore, that Frankel's data are not inconsistent with this conclusion.

The action of erepsin on casein is about as extensive as that of pepsin,[45] but following a peptic hydrolysis erepsin is a very effective agent in disrupting the protein molecule. Frankel has obtained in general a greater cleavage of proteins in a peptic-ereptic hydrolysis than in a peptic-tryptic hydrolysis.

It is a difficult, probably an impossible, task to demonstrate that proteins are absorbed only as amino acids; the evidence that practically complete hydrolysis is possible and is perhaps the normal course of events, seems clear and has been well summarized by Abderhalden as follows:

1. The intestinal tract is provided with enzymes which are capable of hydrolyzing proteins quickly and completely.
2. All of the amino acids known to occur generally in the protein molecule have been found in the intestinal contents during digestion, and particularly those which are not liberated from proteins until the last stages of hydrolysis (*i.e.*, proline and phenylalanine).
3. Animals seem to be just as well nourished when fed mixtures of amino acids, including all those that the animal cannot itself synthesize, as when fed proteins.
4. The processes of metabolism are more closely related to amino acids than to any other of the hydrolytic products of protein.

THE ABSORPTION OF AMINO ACIDS

The absorption of the products of protein digestion is probably entirely insignificant in the stomach, but as soon as the chyme leaves the stomach and enters the duodenum, it passes into a region of very active absorption. Here the amino acids are liberated by enzymic cleavage and here the mucosa lining the alimentary tract is especially adapted to the

function of absorption. Its surface area is greatly increased by the presence of the villi, small finger-like projections into the lumen of the intestine, giving to its mucosa a velvety appearance and feel. The axis of each villus is occupied by a small lacteal, or lymphatic vessel, which is in turn surrounded by a capillary network. Into these small lymphatic and blood vessels the end-products of digestion are absorbed; the active agents of absorption are the epithelial cells covering the villus in a single layer membrane, continuous with the intestinal mucosa and the epithelial lining of the intestinal glands. Beneath the mucosa of the villus a few longitudinal unstriated muscle fibers are found, whose function is to bring about the movement of the villus during digestion. The movements of the intestinal villi have been studied by Hambleton,[50] who found that they are independent of intestinal peristalsis and are stimulated by the presence of nutrient material in the intestine. During active digestion and absorption, the villi exhibit movements of two kinds: (a) a lashing movement, which may be supposed to aid in the mixing of the intestinal contents and thus promote the action of the digestive juices as well as the processes of absorption, and (b) an alternating retraction and extension, a form of movement which may be of special value in absorption, particularly absorption through the lacteals, by aiding in the more active circulation of blood and lymph. The villi are largest and most numerous in the duodenum and jejunum, and become gradually smaller and fewer in the lower portion of the small intestine.

BIOLOGICAL EVIDENCE OF THE ABSORPTION OF INTACT PROTEIN: THE ANAPHYLACTIC REACTION

It is a matter of general physiological interest and considerable pathological significance that intact proteins may be absorbed from the alimentary tract of animals, albeit only in minute amounts. Macallum [51] has observed microscopically the absorption of egg yolk proteins by the intestinal mucosa of guinea pigs and rabbits. A most interesting demonstration of the absorption from the intestinal tract of dogs and men of an intact protein and its excretion by the kidney is afforded by some experiments of Mills and associates [52] on tissue fibrinogen. Tissue fibrinogen is a powerful blood coagulant obtained from animal tissues, consisting of a compound of a phosphoprotein and a phospholipin in about equal proportions.[53] Its characteristic effect in quickening the clot-

[50] Hambleton, B. F., *Amer. J. Physiol.*, 1914, xxxiv, 446.
[51] Macallum, A. B., *Proc. Amer. Soc. Biol. Chem.*, Dec., 1923, xvii.
[52] Mills, C. A., Dorst, S. E., Mynchenberg, G., and Nakayama, J., *Amer. J. Physiol.*, 1922-23, lxiii, 484.
[53] Mills C. A., *J. Biol. Chem.*, 1921, xlvi, 135.

ting time of blood is noted even in very dilute concentrations, and is rapidly destroyed by tryptic digestion. In human subjects, the presence of tissue fibrinogen in blood drawn from the finger tip could be detected two and one-half minutes after swallowing a portion of this protein. Kidney excretion of the coagulant occurred in dogs after placing the substance in an intestinal loop, though such excretion was not observed in men after oral administration.

The presence of unchanged protein in the blood after its ingestion by mouth has also been demonstrated by the use of precipitin tests or of the anaphylactic test. Thus, Van Alstyne and Grant,[54] using the anaphylaxis method, demonstrated egg albumin in the blood of dogs one and one-half hours after placing it in a loop of intestine. Results similar in significance but obtained in a slightly different way are cited by Wells.[55] The anaphylactic effect is ordinarily obtained after sensitizing an animal to a definite protein by injection of small quantities in the blood. After such injection, it takes about 10 to 14 days for a condition of sensitization to develop. After such time, a second injection of the same protein will induce anaphylactic shock, the symptoms of which differ in character and severity in different animals, but involve generally a bronchoconstriction and vascular and vasomotor disturbances. The characteristics of the reaction significant in this connection are, first, its strictly specific nature, a definite protein sensitizing an animal to itself only, not to proteins in general, and, second, only native proteins induce this effect.

Repeated injection of a protein will first produce a condition of sensitization and finally a condition of antisensitization. However, Wells found that a condition of antisensitization could be induced by feeding definite proteins as well as by injecting them into the blood. Thus, guinea pigs raised on a ration containing oats cannot be made to show an anaphylactic reaction to oat proteins. This development of a condition of antisensitization or immunity to anaphylaxis to proteins occurring in the food may, with guinea pigs, take two or three months, and may vary in point of time for different proteins; for example, it seems difficult if not impossible to immunize guinea pigs to milk proteins even by long continued milk feeding. This is probably due to a difference in the permeability of the intestinal mucosa to unchanged proteins, if one may judge from the results of an experiment of Hektoen, Kanai, and Dragstedt[56] on dogs. After feeding fasting dogs beef proteins, no trace of them could be found in the portal or systemic blood by means

[54] Van Alstyne, E. V. N., and Grant, P. A., *J. Med. Res.*, 1911, xxv, 399.
[55] Wells, H. G., *Physiol. Rev.*, 1921, i, 44.
[56] Hektoen, L., Kanai, P. H., and Dragstedt, L. R., *J. Amer. Med. Assoc.*, 1925, lxxxiv, 114.

of the precipitin test; however, after feeding beef thyroids, beef thyreoglobulin was detected by the same test in the portal and systemic blood and occasionally also in the urine.

Such results as these can only be interpreted to mean that, even under normal conditions of feeding, small amounts of food proteins, either constantly or intermittently, may find their way unchanged into the blood. A continual inflow of minute traces of food protein into the blood will naturally immunize the animal organism against such proteins. However, with some individuals and some foods this immunization or antisensitization does not readily supervene, but rather a condition of sensitization with respect to some one or a few food proteins persists, so that the ingestion of such foods results in the production of anaphylactic symptoms. This state of affairs probably accounts for many cases of so-called idiosyncrasy to a food, such as egg white, and certain types of asthma seem to be simply an exaggerated anaphylactic response to some particular food protein to which the individual has been sensitized by previous feeding.

TOXIC EFFECTS OF PROTEINS AND THEIR DERIVATIVES

It is a significant fact that proteins and many of their intermediate products of hydrolysis exert profound physiological effects on animals if they gain access to the blood or the tissues. The anaphylactic reaction of proteins has just been touched upon. The so-called "proteose" or "peptone" intoxication, characterized by a marked fall in arterial blood pressure, a marked retardation of the clotting time of blood and an increase in protein catabolism, may be referred to in this connection, while Vaughn [57] and his co-workers have shown that a very toxic body can be prepared by digesting any true protein with an alcoholic solution of sodium hydroxide. While the symptoms produced by the injection of Vaughn's "crude soluble poison" are similar in nature to those of "proteose" intoxication,[58] they must be ascribed to some substance or substances other than proteoses, since they result from dosages much smaller than the effective dosage of "proteose." It is evidently an extremely important function of the intestinal mucosa to prevent the passage of these products into the portal blood or the lymph. In this connection the erepsin content of the intestinal mucosa is evidently of high significance, since protein derivatives (proteoses) do accumulate in the mucosa during digestion.[59]

[57] Vaughn, V. C., "Protein Split Products in Relation to Immunity and Disease." Philadelphia, 1913.
[58] Underhill, F. P., and Hendrix, B. M., *J. Biol. Chem.*, 1915, xxii, 465.
[59] Abel, J. J., Pincoffs, M. C., Rouiller, C. A., *Amer. J. Physiol.*, 1917, xliv, 320.

The relation of these protein intoxications to the amino acid make-up of the proteins has been the subject of much speculation with no unanimity of opinion. As Underhill says, "almost every study along this line points to a different amino acid as the cause of the peptone effect." There can be little doubt that the amino acid make-up of the protein bears some relation to its intoxicating effect, since gelatin, a markedly "incomplete" protein, exerts no anaphylactic effect, and on partial hydrolysis produces proteoses with no physiological action. Similarly the proteoses from zein, another "incomplete" protein, although they produce physiological effects analogous to those of typical proteoses, are much less toxic.

However, amino acids themselves are physiologically inert. The different protein intoxications or "shocks" must therefore be due, directly or indirectly, to the linkage of amino acids together in such a way that some chemical radical, whose presence neutralizes the pharmacological action of some other radical or larger chemical grouping, is itself neutralized. It is a significant fact that the simple decarboxylation of amino acids, yielding amines, results in the production of substances possessing a greater or less physiological activity.[60] In particular, the decarboxylation of histidine produces an amine, histamine, possessing a powerful physiological activity and producing reactions similar in many respects to anaphylactic and peptone shock. The inactivity of histidine is apparently to be ascribed to its carboxyl group. Arai [61] has recently shown that esterification of the carboxyl group of histidine results in the production of an ester reacting similarly, though not as powerfully, as histamine. The hydrochloride of the methyl ester of histidine was found capable of causing a fall in the blood pressure of animals and of stimulating smooth musculature. However, the limiting concentration of the ester required to stimulate a piece of guinea pig uterus is 1 : 140,000, while that of histamine is 1 : 250,000,000. The ethyl esters of tyrosine, phenylalanine, cystine, and glycine were all found to be more or less active physiologically.

Proteins and their derivatives are known to consist of amino acids joined together generally in an acid amide fashion; however, as Abderhalden has pointed out, an ester-like coupling of amino acids, involving a carboxyl and a hydroxyl group, is possible. In view of the experimental results of Arai, the thought is suggested that indifferent amino acids could become toxic as the result of the manner of their linkage together, and that toxic proteins and protein derivatives may owe their

[60] Guggenheim, M., "Die biogene Amine." Berlin, 1920.
[61] Arai, M., *Biochem. Z.*, 1923, cxxxvi, 202.

toxicity to ester-like couplings in their molecules. Therefore, the fact [62] that histamine-free peptone may induce typical peptone shock in animals may not indicate any essential difference in the mechanism of peptone and histamine activity.

THE ACTION OF INTESTINAL BACTERIA ON AMINO ACIDS

The actual decarboxylation of amino acids by the action of bacteria normally inhabiting the alimentary canal of animals is an established fact.[63] The bacterial reactions relative to amino acids that have been investigated the most extensively are decarboxylation and deamination. According to Hanke and Koessler the production of amines from amino acids by bacteria occurs only in acid-producing media and may be looked upon as a protective mechanism resorted to when the accumulation of H-ions within the bacterial protoplasm is incompatible with its normal life processes. The amines, according to this conception, can be thought of as reaction buffers. On the other hand, deamination results in a buffered or alkaline medium. Thus, colon bacilli in an acid medium will produce tyramine from tyrosine, while in an alkaline or a buffered medium, they will produce phenol. In an acid medium, histidine is changed to histamine, but in a buffered or alkaline medium histidine is either not catabolized, or it is catabolized with the production of products not containing the imidazole ring.[64]

The action of intestinal bacteria on amino acids or partially digested proteins carried through to the large intestine (probably first hydrolyzed by bacterial proteases) include a long list of amines, fatty acids, hydroxy-acids, phenols, and alcohols, that have been identified either in the intestinal contents or in the urine in which they have been excreted after absorption and detoxication of one kind or another. Particular interest has centered around the products of bacterial action on tryptophane, *i.e.*, indole, indole ethyl amine, or, less commonly, skatole, or products closely related to these, due largely to the color reactions permitting their ready determination and the study of their formation. From cystine, bacteria produce methyl mercaptan and hydrogen sulfide, and possibly also diethylsulfide. Ammonia is, of course, a characteristic product of putrefaction.

THE PATHOLOGICAL SIGNIFICANCE OF INTESTINAL PUTREFACTION

Many of the products thus formed are relatively harmless to the animal organism, though many of them are capable of producing pro-

[62] Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1920, xliii, 567.
[63] Mellanby, E., and Twort, F. W., *J. Physiol.*, 1912, xlv, 53. Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1919, xxxix, 539; 1922, 1, 131; 1924, lix, 835, 855.
[64] Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1924, lix, 867.

found physiological effects, as testified to by the special defense mechanism, generally supposed to be centered in the liver, against the possibility of their obtaining access to the blood. In spite of this defense mechanism, however, more or less direct and convincing evidence has been adduced connecting certain pathological conditions or symptoms with excessive putrefaction in the large intestine. Among such symptoms may be mentioned certain skin eruptions, certain types of anemia, foul breath, and even temporary sterility in women.

However, it is very easy to exaggerate the case against the harmful results of intestinal putrefaction and to magnify a mere possible or even reasonable explanation of pathological conditions into a positive diagnosis. Until recently, for example, the symptom-complex commonly designated as autointoxication, coincident with intestinal stasis or chronic constipation, was more or less generally, and quite reasonably, explained as the result of the absorption of toxins from the intestinal tract, particularly the absorption of histamine. However, though histamine is probably normally formed in the intestine and probably formed in increased amounts as a result of intestinal stasis, Koessler and Hanke [65] conclude that their experimental results on the absorption and detoxication of histamine may be explained fully on the assumption that it is rendered pharmacologically inert in its passage through the intestinal wall. Meakins and Harington [66] were more successful in demonstrating an absorption of histamine from different levels of the intestine, if one considers a concomittant fall of blood pressure indicative of such absorption, but they consider, nevertheless, that the balance of evidence obtained is against the view that histamine is an active agent in causing intestinal intoxication, except possibly in those cases where a definite structural deficiency, such as ileo-cecal incompetency, is involved. Both Koessler and Hanke, and Meakins and Harington found that the liver did not exert any marked protective effect against histamine, except a purely mechanical one associated with its extensive capillary network. In the same vein, Donaldson [67] has shown that the symptoms of autointoxication induced by voluntary holding of feces for 100 hours were almost immediately relieved by passage of feces in response to an enema. Such an outcome is quite incompatible with the toxin theory. According to Donaldson the typical symptoms of autointoxication may be primarily explained on a mechanical basis; that is, distention and irritation of the lower bowel by fecal masses, the

[65] Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.* 1924, lix, 889.
[66] Meakins, J., and Harington, C. R., *J. Pharm. and Exper. Therap.*, 1922-23, xx, 45.
[67] Donaldson, A. N., *J. Amer. Med. Assoc.*, 1922, lxxviii, 884.

nervous system being the distributing agency and all tissues sharing in the disquietful state.

THE INHIBITION OF INTESTINAL PUTREFACTION

However, the inhibition of intestinal putrefaction is considered of importance in the control of certain disorders of the intestinal tract and of certain types of dermatitis. There are conceivably two methods of inhibiting putrefaction, one involving the implantation in the intestine of aciduric types of bacteria, and the other involving diet regulation leading to a marked change in the character of the contents of the large intestine such as to favor an aciduric flora. Putrefaction in the large intestine is characterized by the production of a reaction close to neutrality,[68] while carbohydrate fermentation is characterized by a reaction distinctly acid in character.

Attempts to implant aciduric bacteria in the intestine have met with varying success. If the bacterium used is a normal inhabitant of the intestine, such as *Bacillus acidophilus* or *Bacillus bifidis,* implantation may be successful.[69] If it is not a normal inhabitant, such as *Bacillus bulgaricus,* implantation is unsuccessful. Even with the aciduric bacteria suitable for growth under the conditions obtaining in the large intestines, the most successful and persistent implantation involves a diet change leading to the persistence of carbohydrate residues in the large intestine.

The most practicable and effective way of modifying the intestinal flora to reduce putrefaction is to introduce into the diet certain carbohydrates so difficultly digestible or absorbable that they will persist in appreciable amounts throughout the length of the intestinal tract. Readily digestible carbohydrates, such as maltose, glucose, or sucrose, are ineffective, while lactose and dextrin are highly effective.

It appears that in the presence of carbohydrate, protein material or protein degradation products are largely protected from bacterial action except for the anabolic processes essential for bacterial growth and multiplication. This fact, together with the great difference in the products of bacterial metabolism resulting from maintenance on protein or carbohydrate media, is strikingly illustrated by the following quotation from Kendall: [70]

> Two great classes of energy-containing substances are generally available for microbic catabolism, namely, the carbohydrates, and the proteins or their deriva-

[68] Canno, P. R., and McNease, B. W., *J. Inf. Dis.,* 1923, xxxii, 175.
[69] Rettger, L. F., and Cheplin, H. A., "Intestinal Flora." Yale Univ. Press, New Haven, Conn., 1921, Fisher, R. C., *J. Dairy Sci.,* 1920, iii, 414.
[70] Kendall, A. I., *Physiol. Rev.,* 1923, iii, 438.

tives. Oxidizable carbon is contained in each, but the substances resulting from the respective intracellular vital combustions of this carbon are, or may be, widely different. Thus the same organism, utilizing the carbon of a carbohydrate for its energy on the one hand, or the carbon of a peptone on the other hand, may be a veritable Dr. Jekyll or a Mr. Hyde in so far as the results of its activity are concerned. A few examples selected from a multitude of well authenticated instances of this important phenomenon will be illuminating.

The diphtheria bacillus, growing in a broth medium containing peptone and meat extractives, but no utilizable carbohydrate, produces that potent specific soluble toxin known as the diphtheria toxin. The colon bacillus cultivated in the same medium forms indol from the amino acid tryptophan. Bacillus proteus elaborates a soluble proteolytic enzyme under similar conditions. The Shiga dysentery bacillus generates a soluble poison from the same ingredients, which is said to be unlike that formed by any other microbe. Each organism, so it appears, acts characteristically and differently upon the common protein constituents of the broth medium, in that the soluble products resulting from its growth are distinctive.

The same holds in the human body, where the protein of the human tissue furnishes the requisite nutriment. The diphtheria bacillus through its exotoxin incites the clinical entity, diphtheria. The Shiga bacillus is the etiologic agent of one type of bacillary dysentery. In like manner Bacillus coli forms indol in the alimentary canal from the tryptophan of the ingested protein of the food.

A striking change in the character of the products formed by these microbes takes place if some ordinary glucose is added to the respective cultures before inoculation. It must be remembered that the nitrogenous (protein) constituents of the culture medium remain quantitatively and qualitatively the same; the only change is the mere addition of a small amount of glucose. The diphtheria bacillus does not form the potent, soluble toxin in the glucose-protein medium; it produces lactic acid. The Shiga bacillus fails to make its characteristic poison; it also forms lactic acid. Indol is no longer detectable in the glucose broth culture of the colon bacillus, and no soluble proteolytic enzyme can be detected in the filtrate of the glucose broth culture of Bacillus proteus. In place of indol and the enzyme, both the colon bacillus and Bacillus proteus cultures contain acidic products, mostly lactic acid.

This remarkable transmutation of the products formed by the four microbes upon the addition of glucose to their nutritive supply is not apparently accompanied by a transformation of their structural phase; indeed, the microbes are qualitatively indistinguishable when they are tested for their specificity with their specific agglutinins, lysins or precipitins.* The differences, in fact, are directly attributable to the utilization of carbohydrate in place of protein for their energy requirements.

A mass of information has accumulated which indicates very clearly that utilizable carbohydrate added to cultural media protects or spares the protein of the medium from breakdown for energy among the vast majority of microbes, precisely as carbohydrate protects protein in the animal and the human body.

The effects of adding utilizable carbohydrate to cultural media as exemplified in the four bacteria just mentioned are summarized in the following table:

* Simons (*J. Inf. Dis.*, 1915, xvii, 500) has shown, however, that there are quantitative differences in the respective agglutination, precipitation and complement fixation reactions of the plain and glucose cultures.

Organism	Significant product in protein medium	Significant change in the carbohydrate-protein medium
Diphtheria bacillus	Soluble diphtheria toxin	Lactic acid. No toxin
Shiga bacillus	Soluble Shiga toxin	Lactic acid. No toxin
Bacillus coli	Indol	Lactic acid. No indol
Bacillus proteus	Soluble proteolytic enzyme	Lactic acid. No enzyme

It follows that the specificity of chemical action of these bacteria depends upon their utilization of protein for energy.

Conversely, when these bacteria are grown in media containing utilizable carbohydrate, they form lactic acid which is the chemical basis of buttermilk. These two generalizations are appliable to all the bacteria parasitic or pathogenic for man thus far studied with the exception of the very few that do not utilize carbohydrate for energy.

THE EFFECT OF DIET UPON INTESTINAL PUTREFACTION

It is a matter of first importance in practical nutrition to determine the effect of different foods and classes of foods on the intestinal flora. Such determinations are ordinarily made by qualitative bacterial analysis of the fecal flora,[71] or by analysis of the urine [72] for those substances resulting from the absorption of putrefactive products from the intestinal tract, *i.e.*, indican, phenols, or, less certainly, the so-called "ethereal sulfates." Recently Bergeim [73] has introduced a new method based upon the fact that the organisms of true putrefaction presumably responsible for the more severe intestinal toxemias are anærobic in character. The degree to which a readily reducible substance is reduced in its course through the alimentary canal should, therefore, be related to the extent of intestinal putrefaction to which the diet with which it is fed is subjected. The fact that ferric oxide when ingested with certain test foods is reduced to a variable extent in the intestine to ferrous oxide, that it is not absorbed to any appreciable extent, and that both forms are readily estimated in the feces is made the basis of a simple method for obtaining a quantitative index of reduction processes in the intestine, and presumably of intestinal putrefaction. The results that Bergeim has already published are in sufficiently close agreement with results obtained by more direct methods that considerable confidence may be felt in the essential accuracy of the reduction method. Actual examination of the contents of the intestinal tracts of rats subsisting on diets containing 1 per cent of ferric oxide showed that no reduction took place in the stomach and practically none in the upper small intestine; active reduction was practically confined to the cecum and large intestine where putrefactive processes are also confined.

[71] Herter, C. A., and Kendall A. I., *J. Biol. Chem.*, 1909-10, vii, 203.
[72] Underhill, F. P., and Simpson, G. E., *J. Biol. Chem.*, 1920, xliv, 69.
[73] Bergeim, O., *J. Biol. Chem.*, 1924, lxii, 45, 49.

Bergeim found that meat and egg proteins gave rise to high degrees of intestinal reduction in contrast with casein. Vegetable proteins gave intermediate results. The cooking of meat resulted in lessened reduction. Starch, sucrose, glucose, fructose and maltose exerted little effect on intestinal reduction, while dextrin and lactose, which possess an established potency in altering the intestinal flora from the putrefactive to the aciduric type, markedly diminished intestinal reduction. Fat produced little effect. Milk given alone was associated with very low reduction values and, when added to other diets, decreased the extent of intestinal reduction. Fruit and green vegetables tended to give low reduction values. Certain antiseptics tested failed to produce any permanent decrease in reduction, while intestinal stasis in all cases led to marked increases in reduction. The method apparently possesses great value as an index of intestinal conditions relative to bacterial activity, and its results are probably of more general significance than estimations of particular products of bacterial action excreted in the urine, even when the methods available for such estimations are accurate. In interpreting the reduction values obtained with different foods it should, of course, be remembered that several factors contribute to the final result, such as the digestibility of the protein as well as of the carbohydrate constituents, and particularly the effect of the food on intestinal motility.

THE MEASUREMENT OF THE DIGESTIBILITY OF DIETARY PROTEIN

Although the animal body is equipped with a very efficient chemical and mechanical apparatus for the complete disintegration of food protein, it does not necessarily follow that complete disintegration always occurs. In studying the extent of this disintegration the ordinary procedure is to analyze the food or ration to be tested for its content of nitrogen and to feed a constant amount of it to the experimental subject. After a preliminary feeding period of such length that the fecal excretion may be presumed to represent only the experimental ration, a digestion period is undertaken during which the fecal excretion is collected and analyzed. Evidently the length of the preliminary feeding period will vary with the kind of animal, particularly with reference to the anatomy of its digestive tract. The length of the collection period should preferably be at least 7 to 10 days in length, unless an effective method is used for marking off the feces of this period from the feces of the preceding and following periods. From the determination of the nitrogen intake for the period and of the total fecal nitrogen, the percentage of the intake not appearing in the feces (presumably

digested) is computed. This is the ordinary "coefficient of digestibility of protein."

On the basis of such coefficients, it is evident that there are large differences in the digestibility of the proteins of different food materials. The proteins of meat, when not dried or over-cooked, and of milk seem to be very completely digested, to the extent of 95 per cent or more. The proteins of egg white, on the other hand, are only about 85 per cent digestible under the most favorable conditions, and when consumed in the raw, unbeaten condition they are less digestible. The proteins of cereals and vegetables occupy an intermediate position with respect to the completeness of digestion and exhibit a good deal of variation.[74] The proteins of the leguminous seeds are of relatively low digestibility, ranging from 70 to 80 per cent, while the proteins (nitrogen) of many fruits, such as the apple, prune, and strawberry, are extremely indigestible. Much lower coefficients than 70 to 80 are obtained for the proteins of farm roughages, using sheep, cattle, and horses as experimental subjects.

THE SIGNIFICANCE OF THE COEFFICIENT OF APPARENT DIGESTIBILITY; THE METABOLIC NITROGEN OF THE FECES

It is evident, however, after only superficial consideration, that such coefficients are not accurate measures of the digestibility of food proteins. Being based entirely upon nitrogen determinations, they necessarily imply that the food protein accounts for all of the food nitrogen. In so far as the nitrogen of the food is contained in non-protein materials, probably soluble in the main and readily digestible, the coefficients of digestibility obtained will be larger than the actual digestibility of the true protein. However, the nitrogen of the feces is only derived in part from the food. A certain fraction of the fecal nitrogen, the so-called "metabolic nitrogen," is contained in substances originating in the animal body, such as residues of the bile and the digestive juices and cellular material abraded from the alimentary canal during the passage of the food through it. A certain fraction of the fecal material of all animals consists of bacterial cells, mainly dead cells.* In normal adult men consuming an ordinary mixed diet, the average number of fecal bacteria excreted daily has been found to be[75] about 33×10^{12}, the average daily bacterial dry substance about 5.34

[74] Rubner, M., *Arch. Physiol.*, 1918, 53.

* Although the large intestine is the seat of active bacterial growth, most of these organisms are killed in the passage through the distal portions of the colon and the rectum, because of the accumulation of the products of their own metabolism, accentuated by the concentration of the intestinal contents, the absorption of soluble food material, and the antagonistic action of other bacterial species.

[75] MacNeal, W. J., Latzer, L. L., and Kerr, J. E., *J. Inf. Dis.*, 1909, vi, 123.

grams, and the daily bacterial nitrogen 0.585 grams, amounting to over 46 per cent of the total fecal nitrogen. It is questionable whether this bacterial detritus should be classified as undigested food material, though the elements composing it probably are largely of food origin. It is perhaps reasonable to consider the nitrogen of this material as food nitrogen rendered virtually indigestible.

The advisability of correcting coefficients of "apparent digestibility" of protein for the fecal nitrogenous material of body origin may be considered from two standpoints. It may be argued, on the one hand, that the metabolic nitrogen of the feces need not be considered separate from the undigested food nitrogen in calculating coefficients of digestibility of protein, since it represents to a large extent wastage of nitrogen consequent upon the digestion of the food. If the metabolic nitrogen is not classed with the undigested food nitrogen, it is difficult to decide how to classify it more appropriately in studies of the digestibility of protein; that it should be considered an important factor in such studies can hardly be denied. Furthermore, the nitrogen content of a pound of food, minus the *total* fecal nitrogen resulting from its digestion, is a better measure of the value of that amount of food as a source of nitrogen for the animal body than the nitrogen content minus only the undigested food nitrogen; in other words, in the comparison of two foods as sources of nitrogen for an animal, that food causing the smallest excretion of metabolic nitrogen in the feces per gram of food nitrogen, is, other things being equal, the better of the two. It would seem, therefore, that ordinary coefficients of digestibility of protein may possess a greater significance in human dietetics and in the practical feeding of farm animals than coefficients computed on such a basis as to neglect the metabolic products of the feces.

On the other hand, in the scientific study of the utilization of food protein by animals, the more completely the results can be factored into ultimate terms, the more completely and intelligently can they be explained and interpreted. Regardless of the fact that the metabolic nitrogen of the feces is as truly a waste of nitrogen in digestion as the food nitrogen of the feces, it is of great importance in the scientific study of protein utilization to be able to differentiate the two, since the two types of wastage result from the operation of different factors, vary quantitatively in response to different conditions, and are, in all probability, quite dissimilar qualitatively, on the basis, for example, of the amino acids represented by them. In short, the metabolic nitrogen of the feces represents a different *sort* of nitrogen wastage than the undigested food nitrogen.

FACTORS AFFECTING THE EXCRETION OF METABOLIC NITROGEN IN THE FECES

In determining the importance of correcting coefficients of digestibility of protein for the metabolic nitrogen of the feces, information concerning the amount of this excretion and factors affecting it is of value. Such information can be obtained from determinations of fecal nitrogen excretion on rations containing no nitrogen or but small amounts of nitrogen in such forms as would presumably be completely digestible. A compilation of such information for men, taken largely from a table cited by Thomas,[76] is given in Table 17.

TABLE 17. *The Excretion of Metabolic Nitrogen per Day by Human Subjects.*

Exp. No.	N in Food	N in Feces	Authority	Exp. No.	N in Food	N in Feces	Authority
	gms.	gms.			gms.	gms.	
1	4.02	1.23	Sivén	15	0.30	0.78	Rieder
2	3.5	1.75	Klercker	16	0.30	1.13	Martin and Robison
3	2.83	1.24	Sivén	17	0.29	0.54	Rieder
4	2.42	1.33	Landergren	18	0.27	1.14	Landergren
5	2.4	1.47	"	19	0.24	0.46	"
6	2.19	1.28	"	20	0.22	1.50	Renvall
7	2.1	1.02	"	21	0.14	0.65	Tigerstedt
8	1.65	0.58	"	22	0.06	0.96	Landergren
9	1.65	0.22	"	23	...	0.43	Roehl
10	1.36	1.39	Rubner	24	...	1.24	"
11	1.0	0.75	Landergren	25	...	0.45	Thomas
12	0.37	0.87	Rieder	26	...	0.97	"
13	0.34	1.17	Martin and Robison				
14	0.31	1.52	Renvall				

References:

Landergren: *Skand. Arch. Physiol.*, 1903, xiv, 112.
Klercker: *Biochem. Z.*, 1907, iii, 45.
Renvall: *Skand. Arch. Physiol.*, 1904, xvi, 127.
Rieder: *Z. Biol.*, 1884, xx, 378.
Roehl: *Deut. Arch. klin. Med.*, 1905, lxxxiii, 523.
Rubner: *Z. Biol.*, 1879, xv, 198.
Sivén: *Skand. Arch. Physiol.*, 1901, xi, 308.
Tigerstedt: *ibid.*, 1904, xvi, 68.
Martin and Robison: *Biochem. J.*, 1922, xvi, 407.

There is apparently a wide variation in the excretion of metabolic nitrogen in the feces in these experiments on human subjects, not closely related to the variation in the nitrogen intake. One important factor in determining the amount of metabolic nitrogen in the feces is the amount of food consumed. This fact, apparently not universally recognized, is well illustrated by the experimental data obtained on dogs by Rieder [77] and by Tsuboi [78] (Table 18).

[76] Thomas, K., *Arch. Physiol.*, 1919, 242 (Table 13).
[77] Rieder, H., *Z. Biol.*, 1884, xx, 378.
[78] Tsuboi, J., *Z. Biol.*, 1897, xxxv, 68.

TABLE 18. *The Relation Between the Metabolic Nitrogen in the Feces and the Amount of Non-nitrogenous Food Consumed in Experiments on Dogs.*

Weight of Dry Feces	Fecal Nitrogen Per Cent	Fecal Nitrogen Grams	Food Consumed	Authority
grams				
3.0	3.67	0.11	70 grams starch	Rieder
6.0	3.85	0.22	140 grams starch	"
5.8	4.1	0.24	70 grams starch, 12 grams sugar, 50 grams fat	Tsuboi
12.9	4.4	0.57	200 grams starch, 25 grams sugar, 80 grams fat	"

These figures indicate that the excretion of metabolic nitrogen per gram of food may be fairly constant for similar rations. The close relation between the excretion of fecal nitrogen on nitrogen-free diets and the amount of food consumed is further illustrated by the results of Mitchell [79] on rats, presented in Table 19.

TABLE 19. *Influence of the Amount of Food Consumed on the Excretion of Fecal Nitrogen with Rations Practically Nitrogen-Free.*

Rat No.	Average Weight	Daily Food Intake	Daily Excretion of Urinary Nitrogen	Daily Excretion of Fecal Nitrogen Total	Daily Excretion of Fecal Nitrogen Per Gram of Food
	grams	grams	mgms.	mgms.	mgms.
1.....	218	11.32	24.5	24.3	2.15
	213	7.38	28.6	18.3	2.48
2.....	266	14.24	26.6	35.5	2.49
	257	8.67	37.7	21.7	2.51
3.....	162	10.41	13.2	18.9	1.81
	156	6.00	22.2	11.3	1.88
4.....	145	9.61	12.9	21.0	2.19
	139	6.00	17.4	11.3	1.88
5.....	149	8.73	21.0	15.5	1.78
	143	5.93	22.0	10.7	1.78
6.....	69	5.00	8.3	9.5	1.90
	65	3 60	9.3	5.5	1.53

If the fecal nitrogen excretions of human subjects on low nitrogen diets, compiled in Table 17, are computed per 100 grams of dry food consumed, in so far as this is possible from the published data, a greater degree of uniformity is evident than among the results of the total daily excretions. This has been done in Table 20.

With the exception of four or five results, the values in this table for the excretion of metabolic nitrogen in the feces per 100 grams of dry matter consumed range from 0.16 to 0.28 gram, the average of all

[79] Mitchell, H. H., *J. Biol. Chem.*, 1924, lviii, 880.

TABLE 20. *The Metabolic Nitrogen in Human Feces per 100 Grams of Dry Matter Consumed.*

Exp. No.	Length of Experiment in Days	Dry Matter Consumed	N in Food	Total	N in Feces Per 100 Grams Dry Matter Consumed
		grams	grams	grams	grams
1.....	4[a]	463	4.02	1.23	0.27
2.....	6	650	3.50	1.75	0.27
3.....	17[a]	453	2.83	1.24	0.27
4.....	3	495	2.42	1.33	0.27
5.....	3	556	2.4	1.47	0.26
6.....	4	489	2.19	1.28	0.26
7.....	3	469	2.1	1.02	0.22
8.....	7	333	1.65	0.58	0.17
9.....	3	344	1.65	0.22	0.06
10.....	2	759	1.36	1.39	0.18
11.....	4	762	1.0	0.75	0.10
12.....	3	159	0.37[b]	0.87	0.55
13.....	7	672	0.34	1.17	0.17
15.....	3	147	0.30[b]	0.78	0.53
16.....	7	600	0.30	1.13	0.19
17.....	3	485	0.29[b]	0.54	0.11
18.....	3	353	0.27	1.14	0.32
19.....	2	284	0.24	0.46	0.16
21.....	2	400	0.14	0.65	0.16
22.....	2	344	0.06	0.96	0.28
23.....	4	511	...	0.43	0.08
24.....	3	552	...	1.24	0.23
				Average	0.23

[a] Feces not marked off.
[b] Estimated by Thomas.

being 0.23 gram. It is to be noted that three of the most discordant results (Experiments 12, 15, and 17) were obtained by Rieder and are quite anomalous in that the total fecal nitrogen on the largest food intake is distinctly smaller than on the smaller food intakes.

It is reasonable to suppose that the nature of the food with reference to its physical texture, chemical composition, and digestibility would affect the excretion of metabolic nitrogen by modifying the amount of digestive juices secreted, or the extent of contamination of the fecal material with cellular material and mucus abraded from the lining of the alimentary canal. In conformity with this supposition, Mendel and Fine [80] have been able to show with dogs that the addition of small amounts of indigestible non-nitrogenous material, such as agar and bone ash, to a meat diet, may increase the excretion of fecal nitrogen, presumably of metabolic origin only, from 60 to 192 per cent. Mitchell [79] has presented somewhat more direct evidence in that cellulose ingestion by rats subsisting on a nitrogen-free diet was found to increase the excretion of fecal nitrogen. Four rats from the same litter,

[80] Mendel, L. B., and Fine, M. S., *J. Biol. Chem.*, 1912, xi, 1.

subsisting upon a nitrogen-free diet, well-balanced with respect to all nutrients but protein, were allowed access to filter paper in one period and in a succeeding period were not allowed access to filter paper. In the first period a considerable though undetermined amount of filter paper was actually consumed. The data relating to the fecal excretion of nitrogen are given in Table 21.

TABLE 21. *Effect of Roughage on the Amount of Metabolic Nitrogen in the Feces.*

Rat No.	Initial Weight	Final Weight	Food Eaten per Day	Fecal Nitrogen per Day	Fecal Nitrogen per 100 Grams Food With Filter Paper	Fecal Nitrogen per 100 Grams Food Without Filter Paper
	grams	grams	grams	mgms.	mgms.	mgms.
1.....	91	86	4.84	6.0		124
	86	79	4.43	7.9	180	
2.....	88	82	4.26	4.7		111
	82	79	4.21	6.9	164	
3.....	99	95	4.94	6.4		130
	95	90	5.16	8.2	159	
4.....	97	90	4.44	5.5		123
	90	86	4.21	8.0	191	
				Average	173	122

The filter paper ingested increased the total excretion of fecal nitrogen an average of 33 per cent, and increased the excretion per gram of ration consumed an average of 42 per cent. While there is no direct evidence relating to the excretion of metabolic nitrogen in the feces per gram of total dry matter consumed, it is reasonable to suppose that this also was increased by the consumption of filter paper, otherwise the consumption of filter paper must have equaled 42 per cent of the consumption of ration.

It is of particular interest in this connection to investigate the question whether the addition of digestible protein to a nitrogen-free diet will increase the excretion of metabolic products in the feces. The products of protein hydrolysis are known to be stimulants to the digestive glands [81] and it might be supposed that the ingestion of food protein would increase the metabolic products in the feces by increasing the amount of digestive juices poured out on the food in its passage through the alimentary tract. However, Mitchell and Carman [82] have shown with rats that the ingestion of egg and pork does not increase the fecal excretion of nitrogen above that on a nitrogen-free diet, while Mendel and Fine have established the same fact for the dog (Table 9 of

[81] Ivy, A. C., and Javois, A. J., *Amer. J. Physol.*, 1925, lxxi, 583, 591.
[82] Mitchell, H. H., and Carman, G. G., *J. Biol. Chem.*, 1924, lx, 613.

Reference 80). In these experiments, the extractives of meat, shown by Pawlow to be powerful gastric stimulants, had no effect in modifying the excretion of metabolic nitrogen in the feces. Underhill and Goldschmidt [83] have also shown that the addition of ammonium salts of inorganic or organic acids had no effect on the excretion of metabolic nitrogen in the feces of dogs maintained upon nitrogen-free rations. It is a matter of great significance that the ingestion of protein does not alter in any way the excretion of body nitrogen in the feces.

Some of the nitrogenous constituents of the juices secreted into the alimentary canal bear no relation to digestion, being true metabolic excreta. Such, for example, are most of the constituents of bile. To this extent one might expect the amount of "metabolic" nitrogen in the feces per day to be related to the size of animal rather than to the amount of food consumed. A study of this relation may be made from the numerous data secured by Mitchell on rats. Since an important and apparently a predominant factor, determining the amount of fecal nitrogen of body origin excreted per day is the amount of food consumed, the relation of the size of animal to the "metabolic" fecal nitrogen may best be studied by considering the effect of varying body weight on the excretion of "metabolic" nitrogen per gram of food consumed, rather than on the total daily excretion of "metabolic" nitrogen. By expressing the latter per gram of food consumed, the effect of a variable food intake is eliminated. The figures in Table 22 permit a study of this relation on the basis indicated.

TABLE 22. *The Relation Between the Excretion of Metabolic Nitrogen in the Feces per Gram of Dry Matter Consumed and the Body Weight of the Rat.*

Body Weight of Rats	Number of Determinations	Average Metabolic Nitrogen per Gram of Dry Food Consumed
grams		mgms.
40– 60	10	1.70
60– 80	32	1.83
80–100	36	1.88
100–120	38	1.90
120–140	29	1.93
140–160	22	2.01
160–180	23	1.90
180–200	9	1.65
200–220	6	1.92

It is evident that while there is a slight tendency for the smaller rats to excrete less "metabolic" nitrogen in the feces per gram of food consumed than the larger, the effect of size of rat is not considerable.

[83] Underhill, F. P., and Goldschmidt, S., *J. Biol. Chem.*, 1913, xv, 341.

However, a possible relation between the size of rat and the excretion of "metabolic" nitrogen per gram of food consumed may be obscured by the fact that the intake of nitrogen-free food among the various rats may have been roughly in proportion to their body weights. If this were true, then the waste nitrogen from the body as well as the waste nitrogen from digestion (both constituting the metabolic nitrogen in the feces) would be excreted in amounts proportional to the amount of food consumed. This possible obscuring factor may be removed, by relating to the "metabolic" nitrogen in the feces per gram of food consumed, the food consumed per 100 grams of body weight, rather than the body weight itself. This has been done in Table 23,

TABLE 23. *The Relation Between the Excretion of Metabolic Nitrogen in the Feces per Gram of Dry Matter Consumed and the Dry Matter Consumed per 100 Grams of Body Weight.*

Dry Matter Consumed per 100 Grams Body Weight	Number of Determinations	Average Metabolic Nitrogen per Gram of Dry Matter Consumed
grams		mgms.
2– 3	8	3.04
3– 4	18	2.35
4– 5	48	1.92
5– 6	46	1.70
6– 7	31	2.11
7– 8	18	2.00
8– 9	19	1.64
9–10	20	1.59
10–11	4	1.54

from which it is evident that there is a marked tendency for rats consuming food in amounts less than 4 per cent of their body weight, to excrete larger amounts of "metabolic" nitrogen per gram of food consumed, and a less marked though distinct tendency for rats consuming more than 8 per cent of their weight in food to excrete smaller amounts of "metabolic" nitrogen per gram of food consumed. For food intakes between 4 and 8 per cent of the body weight, no progressive change occurs in the "metabolic" nitrogen excreted per gram of food consumed. Apparently, except for abnormally low or abnormally high planes of nutrition, the amount of "metabolic" nitrogen in the feces per gram of food consumed is largely independent of the size of animal or of the plane of nutrition as measured by the ratio of food consumed to body weight. This fact must indicate a relatively inconspicuous proportion of true metabolites among the nitrogenous compounds excreted in the feces on a nitrogen-free diet. Conversely, most of such compounds is related to the actual processes of digestion, both chemical and mechanical. The conclusion seems inevitable, therefore, that the

term "metabolic," applied to the body's contribution to the fecal nitrogen, is not accurately descriptive.

In Table 24 will be found a compilation of a number of results available on the excretion of fecal nitrogen per 100 grams of dry matter consumed on rations containing minimal amounts of nitrogen or, in a few cases, containing forms of nitrogen presumably completely digestible. It appears that, if the ration is low in indigestible non-nitrogenous material, all animals tend to excrete approximately 0.2 gram of "metabolic" nitrogen in the feces per 100 grams of dry matter consumed. The presence of indigestible carbohydrate material in the food raises this basal figure roughly in proportion to the concentration of such "roughage" in the diet. The figures in the table are averages of data relating to man, the rat, the dog, and the pig, for diets containing minimal amounts of "roughage."

TABLE 24. *The Excretion of Metabolic Nitrogen in the Feces per 100 Grams of Dry Matter Consumed for Different Animals.*

Animal	Average Excretion of Metabolic Nitrogen in the Feces per 100 Grams of Dry Matter Consumed	Number of Experiments	Authority
	grams		
Man	0.23	22	See Table 20
Rat	0.19	212	Mitchell
Dog	0.17	2	Rieder [a]
Dog	0.18	2	Tsuboi [b]
Dog	0.35	1	Mendel and Fine [c]
Dog	0.21	6	Underhill and Goldschmidt [d]
Pig	0.26	2	Pfeiffer [e]
Pig	0.26	4	Morgen, Beger and Westhauser [f]
Pig	0.15	7	McCollum [g]
Sheep	0.51	10	Morgen, Beger and Westhauser [h]

[a] Rieder: *Z. Biol.*, 1884, xx, 378.
[b] Tsuboi: *ibid.*, 1897, xxxv, 68.
[c] Mendel and Fine: *J. Biol. Chem.*, 1912, xi, I. The ration included 11 per cent of agar.
[d] Underhill and Goldschmidt: *ibid.*, 1913, xv, 341.
[e] Pfeiffer: *J. Landw.*, 1885, xxxiii, 149. The nitrogen-free ration contained about 30 per cent of filter paper.
[f] Morgen, Beger, and Westhauser: *Landw. Vers.-Stat.*, 1914, lxxxv, 1. In two of these experiments, asparagin was given in addition to the nitrogen-free diet, with no apparent effect on the excretion of fecal nitrogen. The nitrogen ration contained about 10 per cent of nitrogen-free extracted straw.
[g] McCollum: *Wisc. Agr. Exp. Sta. Res. Bull.* 21, 1912, p. 58. The nitrogen-free rations fed to these pigs consisted of starch and salts only.
[h] For literature references, see Note f. The N-free ration in this experiment contained daily per head 285 grams extracted straw, 230 grams starch, 25 grams sugar, 8 grams oil, and 20 grams minerals. In all but 4 of the experiments, variable amounts of blood albumin, presumably completely digestible, were added to this basal ration.

The information thus far discussed concerning the excretion of "metabolic" nitrogen in the feces may be briefly summarized as follows:

The most important factor determining the amount of "metabolic" nitrogen excreted in the feces is the amount of dry food consumed.

Another factor of great importance is the concentration of indigestible carbohydrates in the ration. The greater the concentration of roughage, the greater the amount of "metabolic" nitrogen excreted per gram of dry matter consumed.

The presence of proteins in the ration, or of other materials known to stimulate the secretion of digestive fluids, exerts no appreciable influence on the excretion of "metabolic" nitrogen in the feces.

The size of an animal is not an important factor in determining the amount of "metabolic" nitrogen in the feces per gram of food consumed, provided the animal is not consuming abnormally large or abnormally small amounts of food relative to its body weight.

Animals varying in size from the rat to man excrete an average of close to 0.2 gram of metabolic nitrogen in the feces per 100 grams of dry food consumed, provided the ration contains a minimal concentration of "roughage." This figure may be greatly exceeded on diets containing high concentrations of indigestible carbohydrates.

It appears, therefore, that the so-called "metabolic" nitrogen of the feces consists predominantly of nitrogenous waste products of digestion rather than of metabolism. The erroneous idea implied by the term "metabolic" nitrogen applied to the fecal nitrogen of body origin is perhaps in part responsible for the widely prevalent belief that, even for practical purposes, the coefficient of digestibility of protein should be corrected for the fecal nitrogen of non-food origin. On the contrary, if this fraction of the fecal nitrogen is a function of the physical and chemical characteristics of a food and of the amount consumed, it must represent a nitrogen wastage consequent upon the digestion of the food. For practical purposes, therefore, this wastage may logically be charged against the nitrogen content of the food in the same way as the truly indigestible nitrogen of the food itself. No other disposition of this wastage seems reasonable. It is not a constant that could be added to the maintenance requirement for nitrogen (crude protein), since it varies with the amount of food consumed; however, it is a wastage that must be replaced from the food in the same way as the losses of nitrogen in the endogenous catabolism.

THE DETERMINATION OF THE ACTUAL DIGESTIBILITY OF PROTEIN

In digestion experiments with animals on protein-containing rations, the fraction of the fecal nitrogen that is of body origin may be so considerable that the coefficient of digestibility as ordinarily determined is considerably lower than the actual. In the desire to determine the actual digestibility of food protein, various methods have been proposed

for separating the fecal nitrogen of body origin from the fecal nitrogen of food origin. Determinations of the bile constituents and later of mucin have been used for this purpose, while, more recently, Rubner [84] has devised a method for estimating the amount of fecal nitrogen contained in vegetable food residues based upon the insolubility of the latter in acidified alcohol and a concentrated solution of chloral hydrate, in which, according to Rubner, bacteria, epithelial cells, and like constituents of the "metabolic" nitrogen are soluble. However, the method that is used most generally is that originally introduced by Pfeiffer,[85] involving digestion of the feces by a pepsin solution acidified with HCl. The method has at times been extended to include a subsequent digestion with pancreatin, or trypsin, in alkaline solution.

The rational basis of this method is that the nitrogenous products of body origin in the feces are dissolved by pepsin-HCl, while the nitrogenous products of food origin, having already been subjected to such treatment in the alimentary canal, are insoluble. The method may be criticized on the basis of fact and on the basis of the significance of its results. Evidence has been presented by Morgen, Beger and Westhauser [86] that a considerable fraction (approximately 25 per cent) of the "metabolic" nitrogen in the feces of sheep and pigs is insoluble in pepsin-HCl, and that even a subsequent treatment with pancreatin does not effect complete solution. With rabbit feces obtained on a nitrogen-free ration, from 43 to 50 per cent of the fecal nitrogen was found to be insoluble in the pepsin-HCl reagent. In unpublished work, Mitchell * has shown the same to be true of rat feces.

While definite information on the second assumption involved in Pfeiffer's method, *i.e.*, that the fecal nitrogen of food origin is insoluble in digestive enzymes, seems impossible to obtain experimentally, its accuracy has been seriously questioned by Woodman.[87] The incompleteness of the digestion of food proteins by animals is conditioned, as Mendel and Fine and later Mendel and Lewis [88] have shown, mainly by the accessibility of the protein to the digestive enzymes. In natural food products, the protein constituents are frequently protected from

[84] Rubner, M., *Arch. Physiol.*, 1915, 145.

[85] Pfeiffer, Th., *J. Landw.*, 1883, xxxi, 221; 1885, xxxiii, 149; 1886, xxxiv, 425. *Z. physiol. Chem.*, 1886, x, 170, 561; 1887, xi, 1.

[86] Morgen, A., Beger, C., and Westhauser, F., *Landw. Versuchs-Stats.*, 1914, lxxxv, 1.

* A quantity of feces was obtained from rats that had been upon a nitrogen-free ration for 4 days. The feces were dried at a low temperature, ground, and passed through a 40 mesh sieve. After 24 hrs. digestion with pepsin-HCl, 41 per cent of the nitrogen remained insoluble. Subsequent digestion for 15 hours with pancreatin reduced the insoluble fraction to 30 per cent. The enzyme preparations used were from the Digestive Ferments Co. For a further discussion of the metabolic nitrogen in the feces, and its effect on the coefficient of apparent digestibility, particularly as these subjects relate to the nutrition of farm animals, see Mitchell, H. H., The determination of the protein requirements of animals and of the protein values of farm feeds and rations, *Bull. Natl. Res. Council*, 1926, xi, part 1, No. 55.

[87] Woodman, H. E., *J. Agr. Sci.*, 1924, xiv, 428.

[88] Mendel, L. B., and Lewis, R. C., *J. Biol. Chem.*, 1913, xvi, 19, 37.

enzyme action by indigestible carbohydrate structures, or by fatty material. Furthermore, the movement of the food through the alimentary canal is regulated to some extent by its physical texture and its chemical composition. Differences in the rate of passage of different foods may thus determine differences in their digestibility by varying the time of digestion in the different segments of the canal. This implies that some foods may not have been as completely digested by the gastric and pancreatic proteases when they reach the rectum as may be possible. Also, in its passage through the large intestine, much of the material that has protected the food protein from enzyme hydrolysis may have been removed by bacterial action. Pfeiffer's method involves, therefore, a further proteolytic digestion under more favorable conditions than obtain in the proximal segments of the intestine. It is erroneous to assume that in this second digestion no food protein goes into solution.

Differences between species or between individual animals in the completeness of digestion of food proteins may be due to differences in the effectiveness of their digestive juices, or they may be determined by differences in the degree to which the food proteins are rendered accessible to the digestive juices by mastication and other mechanical means and by fermentation by microörganisms resident in the alimentary canal. Since the digestive juices of normal animals, in so far as they have been investigated, appear to be similar in composition and enzyme content, the latter factors relating to the removal of protective materials are probably the most important. However, by subjecting the feces to enzyme proteolysis, the influence of these factors is minimized, and differences actually existing between species or between animals in the effectiveness of their digestive equipment are obscured. Coefficients of digestibility for proteins are obtained which are too high and which do not truly reflect the capacity of the animal for digesting food protein.

The moderate agreement which is sometimes noted between the protein digestion coefficients corrected by Pfeiffer's method and those obtained by direct action of pepsin-HCl on the food or feed itself has been frequently cited [89] as a confirmation of Pfeiffer's method. However, as Woodman points out, it might be urged that such occasional agreement is not altogether unexpected, since the controlling factor in both cases is the reaction of pepsin-HCl with foodstuff or feces. If the feed is prepared for *in vitro* digestion in such a way that its protein is as accessible to the enzyme reagent as is the residual feed protein in the feces resulting from animal experimentation, then a fair degree of agreement may be expected. Such an agreement, however, would be

[89] In this connection the work of Kühn, of Kellner, and of Bülow, cited by Morgen, Beger and Westhauser,[86] in a discussion of their own experiments, may be mentioned.

merely fortuitous and could not be assured by any deliberate control of conditions.

For these reasons, Pfeiffer's method of determining the true digestibility of the proteins of foods or feeds, and other methods based upon the same principles, cannot be recommended. The assumptions upon which they are based cannot be granted and the information obtained is not the information desired.

The relative inherent digestibility of the proteins of a food material may be most directly and satisfactorily investigated by enzymic digestion *in vitro*. The method devised by Waterman, Jones and Johns [90] has yielded interesting results, among which may be mentioned the favorable effect of cooking on the digestibility of the proteins of many legume seeds, the relative indigestibility of the globulin of the peanut (arachin), and the inhibiting effect of gossypol on the digestibility of cottonseed proteins.

The actual digestibility of food protein in the alimentary tract is more difficult to determine in the absence of reliable methods of distinguishing food nitrogen from body nitrogen in the feces. Mendel and Fine [80] propose the following method involving a principle similar to that used by Tsuboi in earlier work:

1. Determine the volume and nitrogen of feces resulting from the material under investigation.

2. Determine the fecal nitrogen resulting from a nitrogen-free diet to which has been added an amount of indigestible non-nitrogenous matter that will yield approximately the same volume of feces as was obtained in (1).

3. Subtract the fecal nitrogen of (2) from that of (1). This excess of nitrogen is presumably due to undigested or unabsorbed nitrogenous matter of the food material.

Such an indirect method seems to offer the best promise of success. Investigations of the effect of increasing percentages of indigestible carbohydrates on the volume and the nitrogen content of the feces per unit of dry matter consumed with different species of animals consuming nitrogen-free diets would in all probability yield valuable information in this connection. It may prove to be true that the "metabolic" nitrogen excreted in the feces per 100 grams of dry matter consumed is approximately the same for rations of equal fiber content, so that the true digestibility of a food or feed may be readily estimated from its apparent digestibility by the use of factors obtained from such investigations.

[90] Waterman, H. C., and Johns, C. O., *J. Biol. Chem.*, 1921, xlvi, 9; Waterman, H. C., and Jones, D. B., *ibid.*, 1921, xlvii, 285; Jones and Waterman, *ibid.*, 1922, lii, 357; 1923, lvi, 501.

CHAPTER V

GENERAL AMINO ACID METABOLISM

I. ABSORPTION AND ANABOLISM

ABSORPTION

During the passage of the food through the alimentary tract, there is a constant production of soluble and diffusible substances, which are absorbed into the circulatory system and transported to the organs and the tissues of the body. It seems probable that only soluble substances are absorbed to any considerable extent, since solutions are readily taken up and the entire digestive process is directed toward the dissolving of the solid constituents of the food. The processes of osmosis and diffusion seem to be largely instrumental in bringing about the absorption of the end products of digestion. The tonic contraction of the walls of the digestive tube, by exerting pressure on its contents, also favors absorption of soluble and diffusible material. By this means the chyme in the intestine is constantly being subjected to filtration under pressure, the filtrate passing through the mucous membrane into the blood or lymph capillaries, and the material to which the mucous membrane is impermeable remaining in the intestinal lumen to undergo further digestive changes or to be excreted unchanged in the feces.

It is clearly evident, however, that these physical processes cannot account for all the phenomena of absorption from the alimentary canal. For example, glucose is absorbed almost as rapidly as sodium chloride and quite as rapidly as sodium iodide, though its diffusibility is very much less than that of either of these salts. The relative impermeability of the intestinal walls to magnesium sulphate is largely responsible for its purgative properties, though this salt is readily soluble in water and diffusible through inert animal membranes. It has been shown that if an animal's own serum be introduced into a loop of its intestine, the serum undergoes absorption. In this case the fluids on both sides of the intestinal mucous membrane are almost identical, so that osmosis could not produce a passage of material from either side to the other. The ready absorption of the serum must indicate a truly secretory activity of the epithelial cells of the intestinal mucous membrane. Thus, absorption from the lumen of the gastro-intestinal canal undoubtedly involves

the activities of the living cells making up its lining membrane. These cells secrete material from the intestine into the blood or lymph, generally in coöperation with the purely physical forces of osmosis, diffusion and filtration, though even in opposition to these forces under certain conditions.

There is no absorption of the products of digestion by the mucous membranes of the mouth or the esophagus. Absorption takes place from the stomach and from the small and large intestines. The amount of absorption of the products of digestion taking place in the stomach is small under normal conditions. Experimentally, the absorption of sugars, amino acids and other soluble compounds from the stomach can be demonstrated, but in normal digestion it probably does not occur at all, or at most in but small amount. In the small intestine absorption takes place rapidly and most of the digested food products are absorbed in this section of the alimentary tract. In the large intestine are absorbed some of the food residues passed on from the small intestine, the products of bacterial action, and a large amount of water.

THE VILLI OF THE SMALL INTESTINE

The wall of the small intestine is lined with numerous conical, round, or club-shaped projections known as "villi" which greatly increase the absorbing surface. The villi are largest and most numerous in the duodenum and jejunum, and become gradually smaller and fewer in the ileum. In the upper part of the small intestine of man there are about 10 to 18 villi per sq. mm. of surface and in the ileum from 8 to 14. In this manner each sq. mm. of intestine represents an absorbing surface of 3 to 12 sq. mm. Not allowing for the surface area of the villi, it is estimated (Sussdorf) that the absorptive surface of the intestine of the horse is about 12.0 square meters, that of the ox 17.1 square meters, that of the pig 2.8 square meters, that of the dog 0.52 square meter, and that of the cat 0.129 square meter. The actual absorptive surface is probably 2 or 3 times these values.

A villus consists of a finger-like prolongation of the mucous membrane. It is covered by a layer of columnar epithelial cells which are the active agents in absorption. Some of these cells secrete mucus. This epithelial layer encloses a network of blood vessels, one or more lymphatic vessels (lacteals), and a few longitudinal unstriated muscle fibers, which are all supported and held together by reticular tissue.

It has been shown by Hambleton [1] that the villi possess distinct movements of their own which are independent of peristalsis and are

[1] Hambleton, B. F., *Amer. J. Physiol.*, 1914, xxiv, 446.

caused by the presence of nutrient material in the intestine. In a starving animal the villi are motionless and are generally found extended, fallen over in various directions, and covered with a mucus-like substance. During active digestion and absorption, however, they exhibit movements of two kinds: (*a*) A lashing movement, which may be supposed to aid in the mixing of the intestinal contents and thus promote the action of the digestive secretions as well as the process of absorption, and (*b*) an alternating retraction and extension, a form of movement which may be of special value in the act of absorption, particularly the absorption through the lacteals, by aiding in a more active circulation of the blood and lymph.

THE BLOOD CIRCULATION OF THE DIGESTIVE ORGANS

The alimentary tract receives its blood supply from the abdominal aorta. The cœliac artery supplies the stomach, spleen, liver and pancreas with arterial blood, while the mesenteric arteries perform the same service for the intestines. These arteries pass to the different segments of the alimentary canal through the mesentery, subdividing into smaller and smaller branches. Upon reaching their destination they may pass along the surface for some distance, immediately beneath the serous membrane. Occasionally branches from these superficial arteries penetrate the muscular layers to the submucosa, where they subdivide repeatedly and give off numerous branches to the mucosa and the muscular layers. The capillary network in the mucous membrane of the stomach and intestines, especially the capillaries of the villi, receive the absorbed food material. The gastric, duodenal, and intestinal glands receive their nourishment from the rich capillary network in which they are imbedded, and from this abundant blood supply they elaborate their secretions.

Each villus as a rule receives its own small arterial branch. The small veins (venules) arise near the surface of the mucous membrane and near the tips of the villi, and, gathering up the blood of the capillaries as they pass along, they penetrate the submucosa and form a close venous network or plexus there. This plexus also receives most of the venules from the muscular coats, and from it proceed vessels which accompany the entering arterial branches towards the surface, where they run underneath the serous coat and finally strike out through the mesentery. In the thin membrane composing the mesentery, the veins from the different levels of the intestine combine to form the large mesenteric veins, which in turn combine and, after receiving the venous

blood from the stomach, spleen, and pancreas, form the large portal vein which leads directly to the liver.

THE BLOOD SUPPLY OF THE LIVER

The liver receives arterial blood direct from the abdominal aorta by way of the hepatic artery, and a much larger supply of venous blood from the intestines, stomach, spleen and pancreas through the portal vein. The hepatic artery and portal vein enter the liver together and subdivide to form smaller and smaller vessels which feed an intricate capillary system penetrating every part of the organ. From this capillary system, collecting veins emerge and these unite to form larger and larger vessels, which ultimately leave the liver as the hepatic veins. The hepatic veins open into the posterior (inferior) vena cava, which leads directly through the abdominal and thoracic cavities to the right auricle of the heart.

THE LYMPH CIRCULATION OF THE DIGESTIVE ORGANS

In the center of each villus is a lacteal or a network of lacteals, which are the capillaries of the lymphatic system and are so called from the milky appearance of their contents (chyle) during the absorption of food rich in fat. The chyle from these vessels drains into a network of lymph vessels in the mucous membrane. This network is in communication with a similar network of larger lymph vessels in the submucosa, the communication being especially free in the neighborhood of the lymph nodules. From the network in the submucosa, efferent lymph vessels pass through the muscular layers, receiving as they pass the lymph from the intramuscular network, and enter a subserous network of lymph vessels. This is especially well developed at the attachment of the mesentery and conveys the lymph to the lacteals in the mesentery which closely accompany the ramifications of the arteries and veins. The mesenteric lacteals enter the larger mesenteric lymph glands near the back of the mesentery and the vessels from these glands convey the chyle to the cisterna chyli (or receptaculum chyli). The cisterna chyli is situated in the abdominal cavity to the right of the aorta and just inside the diaphragm. It is an irregular thin-walled sac. The thoracic duct leads from the cisterna chyli through the thoracic cavity, crossing from the right to the left side of the median line. It passes forward on the left side of the trachea, bends still farther to the left, and at about the level of the left shoulder opens into the venous system near the junction of the left subclavian vein and the left internal jugular. Thus, the lymph from the gastro-intestinal tract is conveyed to

the large veins opening directly into the heart, without passing through the liver.

ABSORPTION INTO THE BLOOD AND THE LYMPH

The great bulk of the products of digestion are, as stated above, absorbed by the epithelial cells of the villi of the small intestine. Two pathways are open to the products so absorbed. They may enter the blood circulation either directly by way of the capillaries of the villi, the portal vein, the liver capillaries and the posterior (or inferior) vena cava, or indirectly by way of the lacteals of the villi, the intestinal and mesenteric lymph circulation, the thoracic duct, and the anterior (superior) vena cava or common jugular vein. As a general statement it may be said that the products of the digestion of carbohydrates and proteins take the former path, and the products of the digestion of fat the latter, though this separation is not by any means complete, a fraction of the absorbed amino acids and monosaccharoses entering the lacteals and a fraction of the fat entering the blood capillaries. At present it is not known what determines this separation of the paths of absorption and transportation.

THE REGULATION OF THE VOLUME AND COMPOSITION OF THE BLOOD

It appears to be highly necessary for the proper nourishment of the tissues of the body that the composition of the blood be constant within narrow limits. In maintaining this constancy all of the tissues of the body seem to be involved, though to an unequal extent. An excess of any one constituent in the blood will under normal conditions result in the rapid absorption of this constituent by all of the tissues of the body. If, for example, a large amount of glucose or of an amino acid is injected intravenously into an animal, it rapidly disappears from the circulating blood and may be recovered from the tissues by extraction. Similarly after a meal, the absorption of the products of digestion results in a greater or less change in the composition of the blood, depending upon the character and the size of the meal; but in the course of a very short time the blood's composition returns to normal due to the regulatory action of the tissues. In fact, for some food substances, such as sugars, withdrawal from the blood may keep pace with absorption so that the composition of the blood may be unaffected.

A very good illustration of this capacity of the tissues to absorb from the blood with great avidity substances occurring there in concentrations greater than normal is afforded by the effects of the absorption of water from the intestine. It is well known that water

quickly passes through the stomach and is rapidly absorbed from the duodenum. Large quantities of water may be absorbed in this way without any appreciable dilution of the blood occurring. Thus, in an experiment reported by Haldane and Priestley,[2] a man drank 5500 cc. of water between 10:45 a.m. and 7:00 p.m. in 500-1000 cc. portions. Samples of blood withdrawn from time to time showed no dilution at all as indicated by a constant percentage of hemoglobin, though in the same period the kidneys had excreted 5460 cc. of urine. This volume of water, exceeding by about one-third the total volume of the blood, had thus been passed through the circulation without any appreciable dilution of the blood in the process.

In experiments on rabbits, Bogert, Underhill and Mendel[3] have shown that after the intravenous injection of a quantity of dilute saline solution equal to the calculated blood volume of the animal, complete restoration of the original blood volume takes place within 30 minutes, even if the kidneys are removed from the circulation by ligation. Evidently the tissues may temporarily store large quantities of water. In these experiments it was shown that this capacity of the tissues to absorb fluid is approximately four times the blood volume of the animal.

It appears from such evidence that the volume of the blood is one of the most important constants of the body, the maintenance of which constitutes one condition for health. Any considerable increase in the volume of blood must inevitably throw an increased burden on the heart, and produce an increase in blood pressure. Therefore, the absorption of large volumes of water from the intestines results in a rapid though temporary storing of this liquid in the tissues rather than in the blood. The excess water above tissue requirements appears then to be more slowly removed from the body by the kidneys.

THE ABSORPTION OF THE PRODUCTS OF PROTEIN DIGESTION

The products of protein digestion, mainly if not entirely the amino acids, are absorbed largely into the blood, though there is evidence that the lymph also is capable of taking up small amounts of these products. During their passage through the intestinal wall the amino acids suffer no appreciable change. The older theories that the amino acids were synthesized into blood proteins in the intestinal walls or were deaminized there have been either pretty thoroughly disproved or have become superfluous. They were originally put forward to explain the failure of chemical methods to detect amino acids in the blood. With the perfection of

[2] Haldane, J. S., and Priestley, J. G., *J. Physiol.*, 1915-16, l, 296, 304.
[3] Bogert L. J., Underhill, F. P., and Mendel, L. B., *Amer. J. Physiol.*, 1916, xli, 189, 219, 229.

methods by which the passage of amino acids from the intestine into the blood could be followed accurately, the necessity of assuming a synthesis of blood proteins was removed. Such a synthesis has always been purely hypothetical, and hence does not need to be disproved before discarding the hypothesis. The demonstration that amino acids resulting from protein digestion pass through the intestinal walls unchanged was foreshadowed by the experiments of Cathcart and Leathes [4] reported in 1906, those of Hohlweg and Meyer [5] in 1908, and particularly those of Folin and Denis [6] in 1912. These investigators showed that during protein digestion, the non-protein nitrogen of the blood other than ammonia nitrogen and urea nitrogen increased, while Folin and Denis showed the same to be true after the injection of individual amino acids into an intestinal loop.

The first successful attempt to follow the absorption and distribution of the amino acids by direct chemical methods was reported by Van Slyke and Meyer [7] in 1912 and 1913. They used the nitrous acid reaction of aliphatic amino groups, which under the conditions prescribed is specific for α-amino acids. The most important results of these investigators relative to the absorption and distribution of amino acids may be summarized as follows:

Analysis of the blood of eight fasting dogs demonstrated the normal presence of amino acids in the blood. Even prolonged fasting for several days does not alter appreciably the amino acid content of blood, which in dogs in the postabsorptive condition averages 3 to 8 mgms. of amino nitrogen per 100 cc. of blood. Even under fasting conditions the amino acid content of blood is subject to considerable variation.

Five hours after a meal of meat, the amino acid content of blood taken from the femoral artery and the mesenteric vein was found to have doubled in value. From the fact that the amino acid content of the blood decreased but little during passage of the blood from the mesenteric vein to the femoral artery, it was concluded that the amino acids are not held back or destroyed by the liver before reaching the other tissues of the body. It appears from this experiment that the amino acids absorbed from the intestine circulate through the entire organism and are offered directly to all the cells of the body.

The fact that the concentration of amino acids in the blood is normally small is accounted for by the rapidity with which the tissues remove them from the blood as soon as they become unusually abundant

[4] Cathcart, E. P., and Leathes, J. B., *J. Physiol.*, 1906, xxxiii, 462.
[5] Hohlweg, H. and Meyer, H., *Beitr. chem. Physiol. Path.*, 1908, xi, 381.
[6] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 87.
[7] Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 1912, xii, 399; *ibid.*, 1913, xvi, 187, 197, 213 and 231.

therein. This is illustrated by the rapid disappearance of alanine when injected intravenously. Twelve grams of alanine dissolved in 400 cc. of water were injected into the femoral vein of a dog weighing 14 kgs. The injection was completed in 5 minutes. In 5 minutes after the injection only about 1.5 grams remained in the circulation, and in 35 minutes, less than 0.5 gram. During this time only 1.5 grams were found in the urinary bladder. Evidently 10 grams of the 12 grams injected were removed by the tissues almost immediately.

Van Slyke and Meyer showed further that the disappearance from the blood of intravenously injected amino acids is the result neither of their destruction by the tissues nor of their synthesis into tissue proteins or into non-protein nitrogenous constituents. The amino acids are merely absorbed from the blood and temporarily retained unchanged by the tissues, from which they may be extracted by hot water. In the case of the muscles a fairly definite saturation point exists, equivalent to a content of 75 to 80 mgms. of amino nitrogen per 100 grams of tissue, representing an increase above the minimal of 50 to 100 per cent. The capacity of the glandular tissue is much more elastic, the saturation point being fully twice as high as for the muscles.

It does not appear, however, that during protein digestion the amino acid content of the tissues is so markedly increased. In fact, it has been concluded by Van Slyke and Meyer [8] and by Wishart [9] that high-protein feeding fails entirely to increase the amino acid content of the tissues. However, the published data on which this anomalous conclusion is based are too few and too discordant to be particularly convincing.[10] Since the data before and after feeding cannot well be obtained upon the same animal, the problem is essentially a statistical one, and, since the amino acid content of the tissues is evidently extremely variable even in fasting, one must be prepared to analyze a considerable number of animals in order to warrant a definite conclusion one way or the other. Upon the basis of the analysis of the tissues of 15 fasted rats and of 18 fed rats killed from 1 to 7 hours after feeding, Mitchell [10] was able to demonstrate a distinct though slight (10%) increase in the amino acid nitrogen of the tissues of half-grown rats during the digestion of a high-protein diet, and a marked increase (25% to 35%) in the tissues of younger rats. The tissues of the younger rats were found to contain higher concentrations of amino acids, ammonia, and urea than the tissues of older rats even in the post-absorptive condition. Luck [10a] has found

[8] Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 1913, xvi, 231.
[9] Wishart, M. B., *J. Biol. Chem.*, 1915, xx, 535.
[10] Mitchell, H. H., *J. Biol. Chem.*, 1918, xxxvi, 501.
[10a] Luck, J. M., *J. Biol. Chem.*, 1928, lxxvii, 13.

that when amino acids are fed to rats in equimolecular amounts, increases of various magnitudes occur in the amino nitrogen content of the liver, but no appreciable change, except with glycine, occurred in the amino nitrogen content of the muscle. In these experiments, although glycine and alanine increased in the same measure the amino nitrogen content of the systemic blood, the former induced a great increase in the amino nitrogen content of the liver, while the later caused no significant change.

Seth and Luck,[11] in experiments designed to throw light upon the specific dynamic action of the amino acids, followed the course of the hyperamino-acidemia subsequent to amino acid administration to dogs. It was noted that there was an earlier and greater increase in the amino nitrogen of the blood and a more persistent circulation at the higher level following the ingestion of glycine or alanine than was observed with several other amino acids tested. In fact, following the ingestion of leucine no appreciable increase in the amino nitrogen content of the blood was observed. This differentiation of glycine and alanine from the other amino acids was shown to be due, in all probability, to an increased rate of absorption from the intestinal tract. It is quite possibly associated with the greater specific dynamic effect of these amino acids.

THE FATE OF THE AMINO ACIDS IN THE TISSUES

Since the solid material of protoplasm is so largely protein in character, as is also much of the intercellular material of tissues, it is evident that the body must depend to a very great extent upon protein material in practical nutrition for its growth and maintenance. This is in fact the main function of dietary proteins in metabolism, though they are available as sources of energy.

Unlike carbohydrates and fats, proteins or the amino acids derived from them, cannot be stored as such by the adult animal organism, except to a very limited extent. By this is meant that no considerable inert deposits of protein or amino acids are formed in the animal body comparable to the glycogen granules or fat globules existing within the protoplasm of cells and available in case of need, though taking no active part in the cellular metabolism. There is undoubtedly always a certain amount of amino acids contained in tissues that can be readily extracted from them by water, but this cannot be considered as a storage of amino acids, since it is not depleted to any considerable extent during fasting, as are stores of glycogen and fat. Such free amino acids of tissues, as Van Slyke has shown,[8] are merely intermediate steps in the construction and breakdown of tissue proteins. They originate not only (*a*) from the

[11] Seth, T. N., and Luck, J. M., *Biochem. J.*, 1925, xix, 366.

amino acids absorbed from the intestinal tract, but also (*b*) from those continually being formed from the breakdown of the protoplasm itself. This being true, the withdrawal of food would remove the first source, but not the second, which might conceivably even be increased. The inert protein material constituting a large portion of the intercellular material of the connective tissues, such as cartilage and bone, or making up the epidermal appendages, such as hoof, horn, hair, and wool, does not seem to be available for use in case of a need for protein arising in any of the active tissues. The only generally recognized instance of a true storage of protein by animals is found in the storage of small amounts of protein in some egg cells, which subsequently serve as food for the developing embryo. Some indications of protein storage in the liver have been reported as the result of histological examinations, but the significance of such results [12] is difficult to evaluate.

While it thus appears highly probable that animals cannot deposit in their bodies inert masses of protein for storage purposes, as the plant is capable of doing, the active protoplasm of some animal cells may be sacrificed to cover the amino acid requirements of others when the food is insufficient for this purpose or when food is totally withdrawn. Thus, in fasting, the skeletal muscles and the glands of the body may undergo much more extensive disintegration than the nervous system and the heart, the former organs apparently nourishing the latter, which are more intimately concerned in the normal functioning of the body. From the fact that muscle fibers may lose so much of their protein constituents without apparently interfering with their physiological efficiency or their cellular integrity, Moulton [13] considers that protein may in fact be stored in muscle fibers. Greene [14] has earlier argued in much the same fashion from the results of his investigations on the biochemical changes occurring in the muscle of the salmon during the fast of spawning migration. However, there is no indication in these investigations that the protein material removed from muscle cells by fasting or undernutrition was not a constituent of actively metabolizing protoplasm and hence cannot be considered as stored protein as the term is ordinarily visualized. The results are no more surprising than those, for example, of Child,[15] who found that planarian worms could be reduced to a mere fraction of their normal size by starvation without apparently interfering with normal functioning or the ability to regain their normal size on realimentation.

[12] Berg, W., *Biochem. Z.*, 1914, lxi, 428; Berg, W., and Cahn-Bronner, C., *ibid.*, lxi, 434; Stübel, H., *Pflüger's Arch.*, 1920, clxxxv, 74; Junkersdorf, P., *ibid.*, 1921, clxxxvi, 254; Noel, R., *Presse med.*, 1923, xxxi, 158.

[13] Moulton, C. R., *J. Biol. Chem.*, 1920, xliii, 67.

[14] Greene, C. W., *J. Biol. Chem.*, 1919, xxxix, 435.

[15] Child, C. M., *Arch. Entwicklungsmech. Organismen*, 1911, xxxi, 537.

Probably the cytoplasm content of many animal and vegetable cells is primarily dependent upon the plant for nutrition.*

THE POSSIBLE METHODS OF DISPOSAL OF DIETARY AMINO ACIDS

If an animal is fed an amount of protein in excess of that required for the growth and maintenance of the body, the excess of amino acids absorbed into the blood is deaminized, as will be explained later, and its nitrogen is excreted in the urine. The non-nitrogenous residue remaining after the removal of the amino groups may be oxidized directly for the liberation of energy, or it may be stored as glycogen, or as fat.

The fate of amino acids absorbed from the intestinal tract may therefore be considered under the following headings:

1. They may be synthesized into tissue proteins and other nitrogen-containing tissue constituents. In the growing animal, new tissues are formed largely from the amino acids absorbed from the blood, and in both growing and adult animals the "wear and tear" of the tissues in the course of the "endogenous metabolism" is compensated for from the same source.

2. They may be deaminized and oxidized directly for energy purposes.

3. They may be deaminized and the non-nitrogenous residue converted into glucose and stored as glycogen.

4. They may be deaminized and the non-nitrogenous residue may be converted into fat, though the experimental evidence for this conversion is not conclusive.

It should be borne in mind that protein metabolism covers a multitude of complex transformations involving eighteen or more different chemical substances, the amino acids. Furthermore, these different amino acids are not definitely known to be interconvertible in metabolism, and the changes which they undergo in the body and their functions in fulfilling the body's nutritive requirements are in many cases peculiar to the individual amino acids. The case is quite otherwise in carbohydrate metabolism, where the monosaccharoses absorbed from the intestine appear to be of equal nutritive value, at least within wide limits, for the simple reason that they are freely interconvertible; also, in fat metabolism there is no definite suspicion but that tripalmitin, tristearin, triolein, and other fats taken in with the food are strictly replaceable in metabolism. We have no reason to think that any one of these substances has a specific and distinctive function in the body. Aside from differences in digesti-

* However, Truszkowski's work (*Biochem. J.*, 1926, xx, 437; 1927, xxi, 1047; 1928, xxii, 198) indicates that the "nuclear-plasmic" ratio as measured by the ratio of purine nitrogen to non-purine nitrogen, is not changed by inanition in cats, dogs, or frogs, as would have been the case if protein reserves had been present in the cytoplasm of these animals.

bility and energy value, the various fats may apparently be considered as being mutually replaceable in nutrition. The amino acids, however, are quite distinct chemically from one another in many cases; some of them cannot be synthesized by the tissues from any material normally present in the body, and seem to have specific functions which cannot be performed by any known substitute.

THE USE OF AMINO ACIDS FOR STRUCTURAL PURPOSES

In the hours immediately following a meal containing protein, amino acids in large quantities may be absorbed into the blood from the intestine, and are then quickly removed from the blood by all of the tissues of the body before undergoing any appreciable disintegration. In other words, the tissues of the body accumulate in the cellular and intercellular fluids a temporary store of amino acids, above the quantity normally present there. The capacity of different tissues for absorbing amino acids from the blood is restricted within rather narrow limits and differs for different tissues. The liver and the kidney are known to possess a much greater storage capacity than the muscles, for example, though even in the former tissues only relatively small amounts can be accommodated, amounting probably at most to 2.5 or 3.0 per cent of the weight of the fresh tissue.

From this temporary storage of amino acids the cells may remove certain of them for the growth of new tissue or for the replenishment of tissue constituents disintegrated during metabolism. A large variety of different amino acids are represented in the tissues after a protein meal, and it seems probable that for the synthesis of new tissue protein, such as that occurring in a growing cell, a certain assortment of amino acids, containing perhaps 10 or 15 or even more different individual substances, is necessary before synthesis can occur. That is, there are certain amino acids that are essential for cell growth and that cannot be manufactured by the cell. In the absence of even one of these indispensable amino acids, no new protein can be formed and therefore no growth of new cellular protoplasm can be brought about. Several of the amino acids occurring in tissue proteins, on the other hand, can be synthesized from other materials, and therefore their presence in the cell is not essential for the building-up of the protein structure of the tissue.

By feeding experiments with purified proteins known to be entirely lacking or very deficient in one or more of the amino acids, some information has been obtained regarding the amino acid requirements of the tissues. Thus, the protein, gelatin, obtained on heating the collagen of cartilage in the presence of water, and known to be lacking in three

well-known amino acids, tryptophane, tyrosine, and cystine, will not cover the amino acid requirements of the tissues either for growth or maintenance, and an animal fed a ration containing no other protein than gelatin will progressively decline in weight and ultimately die if the ration is not changed. Evidently one or more of the amino acids not occurring in gelatin are essential for tissue growth and repair, and if an animal does not receive these amino acids, as when gelatin is the only protein fed, the tissues cannot be maintained. Another protein, the alcohol-soluble protein of corn, zein, contains no tryptophane or lysine, and, like gelatin, cannot support life. An animal fed a ration containing zein as the sole protein cannot maintain its weight, no matter how much ration is eaten. A third protein, gliadin, the alcohol-soluble protein of the wheat kernel, contains an unusually small percentage of the amino acid lysine. Gliadin fed as the sole protein in a ration will maintain an animal for long periods of time, but with an immature animal, no growth results; or at most a very retarded growth.

THE AMINO ACIDS INDISPENSABLE TO ANIMAL LIFE

The supplementing of these incomplete proteins with the missing amino acids has afforded proof of their indispensability to protein synthesis in the animal body. The indispensability of tryptophane has been abundantly demonstrated in this way. The failure of zein as the sole dietary protein to maintain rats at constant weight has been entirely corrected by an addition of tryptophane in experiments reported by Osborne and Mendel,[16, 17] and the deficiencies of gelatin have been shown by Totani in feeding experiments with rats lasting 22 to 31 days to be largely if not entirely removed by an addition of that amino acid.[18] Similarly the indispensability of lysine for growth has been repeatedly demonstrated by its ability to induce growth when added to gliadin rations or to zein rations previously supplemented with tryptophane,[16, 17, 19, 20] as well as by its ability to enhance the value of edestin for growth.[21] In view of the absence of lysine in zein, the experiments demonstrating protracted maintenance on zein + tryptophane might be interpreted to mean that lysine is dispensable for maintenance, were it not for the fact that the rations used contained 28 per cent of "protein-free" milk, which might possibly have contributed minimal amounts of lysine.

[16] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1914, xvii, 325.
[17] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1916, xxv, 1.
[18] Totani, G., *Biochem. J.*, 1916, x, 382.
[19] Lewis, H. B., and Root, L. E., *J. Biol. Chem.*, 1920, xliii, 79.
[20] McGinty, D. A., Lewis, H. B., and Marvel, C. S., *J. Biol. Chem.*, 1924, lxii, 75.
[21] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1915, xx, 351.

By such simple feeding experiments, Osborne and Mendel have also shown that a deficiency in cystine is the limiting amino acid deficiency of casein.[21, 22] The indispensability of cystine for maintenance has been further demonstrated by Lewis [23] in metabolism experiments on dogs, and its indispensability for growth has been clearly indicated by experiments of Finks and Johns [24] on rats, using rations containing as their sole protein constituent the proteins of beans of the genus *Phaseolus*. Muldoon, Shiple, and Sherwin [25] have used a different and possibly a more effective method in attacking the same problem. A dog when poisoned with bromobenzene will, in the presence of an adequate exogenous supply of cystine, detoxicate the poison by conjugation with cysteine to give *p*-bromophenyl mercapturic acid. However, on a diet of carbohydrate and fat, these investigators found that the dog could not produce cysteine from its tissues for detoxication purposes, nor could it produce cysteine from ingested carbohydrate, ammonium salts, and various sulfur compounds; furthermore, the substitution of gelatin for the ammonium salts also could not facilitate cysteine synthesis. Only when cystine itself was added to the diet was the normal detoxication of bromobenzene accomplished. The inability of the animal body to synthesize cystine from other amino acids is thus abundantly confirmed.

The problem of the indispensability for animal syntheses of the various amino acids occurring in food proteins has been approached by still another method; namely, the feeding of amino acid mixtures obtained by the complete hydrolysis of proteins, from which one or more of the amino acids has been largely or entirely removed by suitable chemical manipulations. The success of this method of investigation rests upon the demonstration that animals are able to grow at a considerable rate upon rations containing amino acid mixtures in place of protein. Experimental demonstration of this fact, starting with the work of Otto Loewi in 1902, may be considered satisfactory at the present time. Abderhalden in particular has accumulated evidence in great excess of that required for conviction. His most successful demonstration [26] consisted of a feeding experiment on a dog lasting for 100 days, during which time the animal was maintained in good health and increased 10 kgs. in weight on a diet containing, as its sole source of nitrogen, a mixture of amino acids obtained from the complete hydrolysis of meat. The hydrolysis was effected by enzymes, though the last stages were completed by the use of a 10 per cent solution of sulfuric acid. As thus

[22] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1916, xxvi, 1.
[23] Lewis, H. B., *J. Biol. Chem.*, 1917, xxxi, 363.
[24] Finks, A. J., and Johns, C. O., *Amer. J. Physiol.*, 1921, lvi, 205.
[25] Muldoon, J. A., Shiple, G. J., and Sherwin, C. P., *J. Biol. Chem.*, 1924, lix, 675.
[26] Abderhalden, E., *Z. physiol Chem.*, 1915, xcvi, 1.

finally obtained no trace of polypeptide compounds could be detected in the hydrolysate. By the use of such amino acid mixtures from which various amino acids have been more or less completely removed, Abderhalden has amply demonstrated the indispensability of tryptophane [26, 27] by simple body weight experiments and by nitrogen balance studies. His experiments also indicate that tyrosine is an essential component of a complete diet, since hydrolyzed casein from which tyrosine had been removed by concentration did not maintain a dog at constant weight. The addition of tyrosine to this deficient mixture immediately led to an increase in weight. The results of experiments on amino acid mixtures deficient in histidine, arginine, and cystine were inconclusive.

Totani [18] has attempted to confirm the conclusion of Abderhalden that tyrosine is an indispensable amino acid. However, he was able to obtain considerable growth for 24 days in rats receiving as their sole source of nitrogen a casein digest from which tyrosine had been largely removed by concentration. The growth of a second group of rats was not improved by the addition of tyrosine to this incomplete amino acid mixture. Furthermore, Totani was able to maintain rats for short periods of time on hydrolyzed gelatin, containing only a minimal amount of tyrosine, supplemented only with trytophane. The nutritional value of the ration was not improved by tyrosine additions. In this connection the possibility of the conversion of phenylalanine into tyrosine should be considered, on the basis of the work of Embden and Baldes,[28] who were able to isolate a small amount of tyrosine from blood containing phenylalanine which had been perfused through a surviving liver. Abderhalden, however, was unable to show a substitution of phenylalanine for tyrosine in his feeding experiments on dogs.

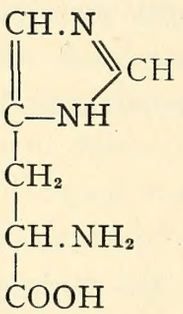
HISTIDINE AND ARGININE

In regard to the question of the ability of the animal body to synthesize the diamino acids, Henriques and Hanson [29] published in 1904 the results of an experiment similar in general character to the experiments of Abderhalden. The ration used contained, besides non-nitrogenous constituents, an enzyme digest of a protein from which the diamino acids were removed by phosphotungstic acid. From the results of one test upon a rat, the conclusion was reached that the amino acid mixture used, deficient in arginine, histidine, lysine, and cystine, was adequate for the maintenance of nitrogen equilibrium and of body weight in the rat. A close study of the data, however, fails to reveal adequate

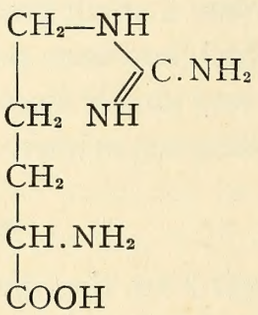
[27] Abderhalden, E., *Z. physiol. Chem.*, 1913, lxxxiii, 444.
[28] Embden, G. and Baldes, K., *Biochem. Z.*, 1913, lv, 301.
[29] Henriques, V., and Hansen, C., *Z. physiol. Chem.*, 1904-05, xliii, 417.

justification for the conclusion drawn, if only because it is impossible to judge of the completeness of removal of the diamino acids in the absence of a description of the chemical manipulations employed, or of a specific test for the diamino acids in the residual mixture.

In 1916, Ackroyd and Hopkins [30] published the results of experiments concerned with the need for arginine and histidine only. Rats were used as experimental subjects, and the nitrogen requirements were supplied by amino acid mixtures obtained from the acid hydrolysis of casein, or mixtures of casein and lactalbumin in equal parts. In all experiments additions of tryptophane and cystine were made to the amino acid mixtures used, since these amino acids would be either partially or completely destroyed during the acid hydrolysis. Feeding experiments showed that the complete amino acid mixture was capable of supporting considerable and continuous growth when embodied in a ration adequate with respect to non-protein nutritive factors. However, when arginine and histidine were removed by precipitation with silver sulfate, the residual mixture was inadequate for maintenance of body weight. Additions of arginine and histidine, either together or singly, restored the growth-promoting property to the deficient amino acid mixture. The experimental results support the conclusions that arginine and histidine are interconvertible in the animal body, and that either one is an essential constituent of a complete amino acid mixture in the nutritional sense. The possibility of interconversion is illustrated as follows:



Imidazole-α-amino-propionic acid (histidine)



Guanidine-α-amino-valerianic acid (arginine)

Since in these experiments a positive nutritive effect was obtained upon the addition of either arginine or histidine to the ration, a demonstration that the basal amino acid mixture contained none of these amino acids after treatment with silver sulfate is quite unnecessary for the interpretation of the results obtained. As the authors state: "The silver method seems to remove both arginine and histidine with great completeness. If traces are left behind, and it is very difficult to disprove this possibility, the fact would not affect the bearing of our experiments,

[30] Ackroyd, H., and Hopkins, F. G., *Biochem. J.*, 1916, x, 551.

which were all comparative and controlled. The presence of traces in the diet of the experimental periods would tell against, rather than in favor of, our main conclusions."

Further evidence for the equivalence of arginine and histidine in nutrition was furnished by Ackroyd and Hopkins from the results of metabolism studies on the rats, in particular the results of the excretion of allantoin. In rats, as in most mammals, allantoin is the chief end-product of purine metabolism. It was found that in rats subsisting upon the diet deficient in arginine and histidine the allantoin elimination decreased from 40 to 50 per cent coincident with the decrease in weight. That the nutritional failure evidenced by the decrease in weight was not the causal factor in the decreased allantoin excretion was shown by the fact that withdrawal of tryptophane was without effect upon the allantoin excretion, although the nutritional failure was even more pronounced than that occasioned by withdrawal of the diamino acids. The point of special interest in these experiments is that the presence of either arginine or histidine in the ration prevented any very great reduction of allantoin excretion, though some reduction (up to 17%) was noted. The results are taken to confirm the conclusion from the body weight experiments that the two diamino acids are equivalent in nutrition, and to suggest that "arginine and histidine play a special part in purine metabolism, probably constituting the raw material (or the most readily available raw material) for the synthesis of the purine ring in the animal body."

Previous to the publication of the work of Ackroyd and Hopkins, Geiling [31] undertook an investigation with a similar object in view. However, adult mice instead of growing rats were used as experimental subjects, and attention was directed toward the amino acid requirements for the maintenance of weight rather than for growth. Enzyme digests of casein from which all of the diamino acids (as well as peptids containing diamino acids) were removed by precipitation with phosphotungstic acid in 5 per cent H_2SO_4 solution, constituted the basal amino acid mixture of Geiling's experiments. The rations used contained 12 per cent of amino acids, 28 per cent of Osborne and Mendel's "protein-free" milk as a source of minerals and vitamin B, butter fat as a source of vitamin A, and various non-nitrogeneous constituents to complete the ration and to disguise as much as possible the sharp taste of the amino acids. The basal monoamino acid mixture, supplemented with cystine and tryptophane, was definitely shown to be inadequate for the maintenance of mice at constant weight, though the ration was consumed in

[31] Geiling, E. M. K., *J. Biol. Chem.*, 1917, xxxi, 173.

adequate amounts. Upon supplementing this basal mixture further with the diamino acids precipitated from gelatin by phosphotungstic acid, a definite increase in weight for 21 days was obtained with four of the five mice in the experiment, the fifth mouse refusing to eat the second ration in an amount sufficient to satisfy its maintenance requirement. The basal monoamino acid mixture plus cystine, tryptophane and arginine, maintained the body weights of 4 mice for 28 days, at the end of which time a substitution of lysine for arginine occasioned a prompt and marked loss in weight and food intake. The basal mixture of amino acids with histidine added in place of arginine was adequate in maintaining the weights of 2 mice for 28 days; at the end of this period, a substitution of arginine for histidine rather improved the nutritive value of the ration than otherwise. With 4 mice that decreased markedly in weight on the basal amino acid mixture plus cystine, tryptophane, and lysine, a substitution of histidine for lysine either checked the loss in weight or induced a slight increase. Inadequate food intakes were probably responsible for the partial success only of this substitution. Therefore, in so far as body weight experiments can demonstrate differences in the nutritive adequacy of rations, Geiling's results may be considered a confirmation of the conclusions of Ackroyd and Hopkins that arginine and histidine are equivalent in nutrition with reference to all animal functions essential to the maintenance of life. It is to be noted in particular that inadequate food intakes do not seriously complicate the interpretation of the data, and that the presence or absence of arginine and histidine in the basal monoamino acid mixture used does not detract from the significance of the results obtained, since the conclusions indicated relate to the indispensability of the diamino acids, not to their dispensability, a distinction clearly stated by Ackroyd and Hopkins.

Abderhalden [32] has recently published the results of experiments on the feeding of definite mixtures of synthesized amino acids to rats and mice. The body weights of the animals are the only experimental data completely reported, though incomplete data on the nitrogen balances are referred to. However, in the absence of complete nitrogen metabolism data it is difficult to judge the significance of these quoted balances. The experiments are important in demonstrating, in feeding periods of 80 to 100 days, the adequacy of a definite mixture of amino acids, containing the following ingredients: glycine, alanine, serine, cystine, α-amino butyric acid, valine, leucine, isoleucine, norleucine, aspartic acid, glutamic acid, hydroxyglutamic acid, phenylalanine, tyrosine, lysine, arginine, histidine, tryptophane, proline, and oxyproline. This is a notable result,

[32] Abderhalden, E., *Arch. ges. Physiol.*, 1922, cxcv, 199.

representing the first successful attempt to replace protein by a synthetic mixture of amino acids.

The results of feeding less complete mixtures of amino acids are unfortunately not always so clear-cut, due to delayed responses to changes in ration and to the shortness of experimental periods (generally 7 to 14 days). The indispensability of tryptophane and cystine is confirmed. A removal of both tyrosine and phenylalanine occasioned a steady loss in weight, while a replacement of either one separately checked the loss or apparently induced a slight gain in weight. The experiment is interpreted to mean that these aromatic amino acids are mutually replaceable, and that one or the other must be present in he ration. The indispensability of lysine, arginine and histidine is also indicated; the replaceability of histidine by arginine was not demonstrated. A factor complicating the interpretation of these experimental results is the variable amount of vitamin B (yeast) offered to the animals. Also, the protein contained in the yeast consumed renders debatable the evidence offered for the dispensability of certain of the amino acids. The fat-soluble vitamins were only occasionally offered to the animals in the form of butter fat.

In 1924, Rose and Cox [33] attempted to confirm the results of Ackroyd and Hopkins relative to the need for arginine and histidine for growth. They duplicated in all essentials the conditions of the earlier experiments and confirmed Ackroyd and Hopkins with reference to the indispensability of histidine. They were, however, unable to obtain any indications that arginine is indispensable or that arginine and histidine are mutually replaceable. Animals declining on an amino acid mixture from which both amino acids had been removed by the method of Kossel and Kutscher, immediately resumed growth when histidine was added to the ration, coincident with a marked increase in food consumption. On the other hand, when arginine was added to the deficient amino acid mixture, no change in the course of decreasing body weight was noted and no improvement in the inadequate consumption of food occurred. Referring to the disagreement between their results and those of the English investigators, Rose and Cox say: "We are entirely unable to reconcile our results with those of Ackroyd and Hopkins. We hesitate to suggest that their arginine preparation may have been contaminated with histidine,* but only with the aid of such an assumption can we under-

[33] Rose, W. C., and Cox, G. J., *J. Biol. Chem.*, 1924, lxi, 747.

* This explanation hardly seems sufficient. In rations containing 24 per cent of fat, Rose and Cox found that a concentration of 0.074 per cent of histidine (0.1 per cent of the monochloride) was necessary for maintenance of weight, while two or three times this concentration was required for slow growth. Ackroyd and Hopkins used rations containing over twice as much amino acids as those of Rose and Cox, but only 12 per cent of fat. Additions of approximately 1 per cent of arginine to the deficient ration induced slow growth. If the

stand their data. Whatever may be the explanation of their findings, the experiments herein described provide convincing proof that *arginine and histidine are not interchangeable in metabolism, and that histidine is an indispensable amino acid.*"

A repetition of the work of Ackroyd and Hopkins on the effect of deprivation of arginine and histidine on the purine metabolism of rats was reported by Rose and Cook [34] in the following year. Quite in line with the experimental data of Rose and Cox, it was shown (*a*) that rats declining in weight on a ration lacking in both diamino acids excrete diminishing amounts of allantoin, while rats declining in weight because of tryptophane starvation show no such reduction in purine metabolism; (*b*) that rats declining in weight on a ration lacking histidine only exhibit a sharp initial reduction in allantoin excretion and then a constancy at the lower level; and (*c*) that rats growing rapidly on a ration lacking in arginine only exhibit an increasing allantoin excretion. The data are taken to warrant the conclusion that "*under ordinary conditions of diet, histidine is a mother substance of allantoin.* If this conclusion is correct, it is evident that one reason why histidine is an indispensable component of the diet is that it is required for nuclear synthesis." Referring to their results with reference to arginine, the authors say: "In contrast to the observations of Ackroyd and Hopkins our experiments show quite clearly that arginine cannot replace histidine in purine synthesis. This result serves to emphasize and reinforce the conclusion of Rose and Cox, that in the absence of histidine from the diet its functions cannot be assumed vicariously by arginine. It appears evident that neither in growth nor in purine metabolism can the two diamino acids be regarded as mutually interchangeable." *

consumption of food in the experiments of Ackroyd and Hopkins was comparable with that in the experiments of Rose and Cox, the arginine of the former investigators would need to be contaminated with 15 to 20 per cent of histidine, in order that the latter should be the active growth stimulant. Since the food consumption might have been greater in the English experiments due to the smaller proportion of fat in the ration, the contamination would not need to be so great, but would still be of such size as to constitute an error readily detectable by a nitrogen determination on the arginine used. Furthermore, the similarity between the quantitative effects of the addition of the arginine and histidine preparations employed by Ackroyd and Hopkins on the excretions of allantoin seems hardly consistent with the assumption that the effect of the former was due merely to a contamination with histidine.

[34] Rose, W. C., and Cook, K. G., *J. Biol. Chem.*, 1925, lxiv, 325.

* Recent experiments by Stewart (*Biochem. J.*, 1925, xix, 1101) concerned with the nutritive significance of histidine and arginine confirm previous investigations relative to the indispensability of histidine, but the results for arginine are midway between those of Ackroyd and Hopkins and those of Rose and Cox, neither indicating the possibility of continuous growth when arginine is substituted for histidine, nor indicating an inevitable and continuous loss in weight. This experimental contribution appears to strengthen the view that differences in food intake may account for the different results obtained in these three laboratories, and that a requisite to the demonstration that a ration is qualitatively inadequate is the assurance that enough of it has been consumed by the experimental animals so that growth would have occurred if the ration were complete. It is significant that the allantoin results of Stewart correlate well with his growth data, just as is true of the results of Ackroyd and Hopkins and of Rose and Cook. Evidently these results are closely dependent upon the growth data and cannot be considered an independent confirmation of them. The reply of Rose and Cox to Stewart (*J. Biol. Chem.*, 1926, lxviii, 217) hardly seems to settle the question at issue. Without changing the plan of their investigations, or even the order in which the rations were fed, they

On the other hand, the significance of variations in the excretion of the end-products of purine metabolism may not be so easy of interpretation. The most obvious significance given to them by Ackroyd and Hopkins and by Rose and Cook may not necessarily be the correct one; in fact, the contradictory character of the two series of experiments with respect to the relation of arginine to allantoin excretion, suggests that variations in the latter may be determined by other factors than the presence or absence in the diet of purine precursors; for example, by variations in the endogenous purine metabolism. The effect of amino acid ingestion in man on the output of uric acid in the urine has been so interpreted,[35] while the recent work of Christman and Lewis,[36] indicating a depression of allantoin excretion in the rabbit by the ingestion of certain amino acids, is but another illustration of the fact that variation in the rate of excretion of urinary constituents is not a phenomenon susceptible of only one interpretation. Returning to the case in point, it is conceivable that purine synthesis might occur from dietary constituents with no immediate effect on the excretion of the end-products of purine metabolism in the urine, while on the other hand, an interpretation of a marked increase in the excretion of allantoin as evidence of the addition to the diet of a purine precursor seems to imply that purine synthesis always occurs in the metabolism of the dietary constituent in question.

A CRITICAL CONSIDERATION OF EXPERIMENTAL METHODS

The experimental data above reviewed indicate that tryptophane, cystine and histidine are indispensable for maintenance and growth, and that lysine is indispensable for growth. As to whether lysine is indispensable for maintenance the data are inadequate for a justifiable decision. The same situation unfortunately exists with respect to tyrosine and arginine.

It seems possible, however, to offer a suggestion to account for the frequent failure of investigators to confirm each other's results. Practically all of the data on amino acid requirements relate to the changes in body weight of experimental animals subsisting on diets presumably complete except for possible amino acid deficiencies. In most experiments no record was reported of the amounts of food consumed, and

report more results identical in significance with their first ones, although with new preparations of basal rations and of amino acid supplements. It would appear that the solution of the question of the dispensability (or otherwise) of arginine in animal metabolism would profit greatly by the application to it of other methods of investigation than those hitherto employed. The authors of this monograph do not feel justified in forecasting the solution of this problem on the basis of available data.

[35] Rose, W. C., *Physiol. Reviews*, 1923, iii, 587.

[36] Christman, A. A., and Lewis, H. B., *J. Biol. Chem.*, 1923, lvii, 379.

in no case was the food intake under control in the sense that for animals and experimental periods being compared the consumption of food was approximately equalized. The failure to consider the amount of food consumed by an animal in judging the nutritive adequacy of an experimental ration seems to be tantamount to assuming that appetite and the other sensations coming from the gastro-intestinal tract determining the amount of food an animal consumes are very nicely adjusted to physiological requirements, in the sense that an inadequate consumption of a ration may be taken as complete evidence of its nutritive deficiency and, conversely, that adequate rations will always be consumed in amounts sufficient to cover the energy requirements. Obviously constituents modifying the flavor or odor of the food may modify its gustatory appeal, and thus indirectly the amount of it that will be consumed, regardless of their value in serving the nutritive requirements of the animal. In this connection may be mentioned the difficulty experienced by Abderhalden [32] in the feeding of rations containing only small amounts of synthetic proline, probably due to traces of decomposition products possessing a pyridine-like odor formed, in spite of all precautions taken, during the concentration of the proline solutions. The different amino acids themselves are known to exhibit differences in taste and odor. Furthermore, it is conceivable that certain dietary deficiencies have a specific adverse effect on the condition (tone) of the gastro-intestinal tract or of its accessory glands, and thus indirectly upon the sensations governing the intake of food. Such deficiencies would presumably be reflected more quickly and more profoundly in diminished food intake than other deficiencies exerting their deleterious effect less directly upon the digestive organs. Also remedying such a deficiency by the proper addition to the diet would possibly effect immediately an increased consumption of food, while remedying other deficiencies only remotely concerned with the digestive system might not be so manifested. The demonstration of Cowgill [37] that vitamin B, more than any other known nutrient, regulates food intake is a case in point. A differentation among the different amino acids in this respect may exist. It appears that histidine, like vitamin B, may be a specific appetite-stimulant; but if arginine is not, the two may still be equivalent in metabolism.

On the other hand, the appetite and related sensations, in the absence of disturbing factors, are known to be potent in equating energy intake with energy requirements,[21] inducing a greater consumption of rations diluted with water or indigestible constituents, and a smaller consumption of rations concentrated in energy by reason of containing large

[37] Cowgill, G. R., *Amer. J. Physiol.*, 1921, lvii, 420; *J. Biol. Chem.*, 1923, lvi, 725.

proportions of fat. Furthermore there is an undoubted tendency for the appetite sensations to differentiate to some extent between adequate and inadequate dietaries, possibly for reasons above given.[38] Just how extensive this ability is with reference to the many different types of dietary deficiencies it would be premature to predict.

These considerations detract from the hypothesis that inadequate food intakes mean inadequate rations, and vice versa. In the absence of any apparent physiological necessity for a causal relation between the amount of a ration consumed by an animal and its nutritive completeness or balance, it appears unjustifiable to assume its existence as the basis of a method of research. While a loose relation of this character undoubtedly exists, with certain types of deficiencies at least, there are too many disturbing factors (mostly unknown in all probability), operating to delay or suppress entirely its manifestation, to permit its use in the interpretation of the results of feeding experiments.

An investigator may naturally feel a high degree of confidence in the significance of experimental results showing uniform consistency in the reaction of his animals to the presence and absence of a given amino acid in the ingesta. However, unless the food intakes are controlled, the evidence is after all of a circumstantial character and cannot be entirely convincing. Moreover, if another investigator working on the same problem with methods identical in all essentials with his own, has also obtained consistent results but of an opposite significance, that fact should cause him to temper the statement of his conclusions. In the case of such a conflict of two sets of data consistent among themselves, it is a fair judgment that a positive result is more significant than a negative one, unless a fair suspicion of the existence of some technical error sufficient to account for the discrepancy in the experiment yielding the positive result would justify a different conclusion. A positive result unexplainable by technical mistakes may be taken to represent a successful experiment, and a negative result a failure due to unknown causes. The first successful attempt to promote growth in animals on a protein hydrolysate or a synthetic mixture of pure amino acids as the sole source of nitrogen is more significant than previous unsuccessful attempts. Similarly an experiment indicating clearly a successful substitution of tyrosine by phenylalanine or of histidine by arginine seems of greater significance than an experiment in which such a substitution could not be made successfully.

In future experimental work on the problem of amino acid requirements, in which the ultimate results will be the changes in body weights

[38] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxv, 19.

induced by adding or subtracting a given amino acid to or from an otherwise complete ration, it would be well to arrive at some decision as to what constitutes a demonstration that an amino acid is or is not indispensable in metabolism. The main criticism of the current method is that, if a change in food intake occurs simultaneously with the addition of a certain amino acid to the ration, or with its removal from the ration, one is unable to decide definitely whether the concurrent change in body weight may not be entirely referable to the change in the amount of food consumed rather than to a change in its composition. In our judgment the demonstration of the indispensability of any amino acid by body weight changes should rest upon the demonstration of a distinct betterment in nutritive condition on rations containing the amino acid than on rations deficient in it, all other conditions having nutritive consequences, *including the amount of food consumed,* being the same.*

The determination of the effect of amino acid additions to and withdrawals from the ration upon the nitrogen balance of experimental animals would seem to be a more direct and hence more conclusive method for the study of amino acid requirements than a mere determination of changes in body weight. The nitrogen balance is a well-established criterion, under certain easily imposed conditions, of the adequacy of the protein or amino acid intake, and does not seem to be affected, at least in the short periods of time required for its determination, by nutritive deficiencies other than those being investigated.[39, 40] On the other hand, the gain in weight of an animal is a somewhat uncertain criterion of protein retention, since it can only occur when all of a series of growth determinants are present in the ration in adequate amounts,

* This argument is discussed in a more general way in an article by one of us that appeared in *Science,* 1927, lxvi, 596. The reader is referred particularly to the subsequent reply of Rose (*Science,* 1928, lxvii, 488), in which exceptions are taken to the conceptions of a rigidly controlled feeding experiment and of the nature and value of negative evidence described in the preceding paper and in this chapter of the monograph. Rose rejects the belief that the animal appetite is infallible in adjusting food intake to bodily requirements; but he does believe that "when an added dietary component leads to an appetite stimulation the explanation is to be found . . . *in the influence exerted by the substance* upon the cells themselves. . . . *Thus our view places the emphasis upon the cell processes* rather than upon the imperfect outward manifestations. It recognizes the fundamental and irrefutable fact that the animal organism is unerringly accurate in its syntheses. If a tissue is to be formed at all, every component required must be available or capable of being manufactured by the cells; otherwise the synthesis will not occur. If growth follows the addition of an essential constituent to an inadequate diet, it does so because cell reactions which could not proceed in the absence of the added factor are now made possibl.e We believe that as the growth syntheses occur, the demand for raw materials is reflected in a stimulation of the appetite resulting in greater food consumption."

The result on the appetite of an animal of adding an essential dietary constituent to its inadequate diet may very well be, in general, as Rose here depicts, though it may be questioned whether this result will inevitably appear in all such cases. It is less obvious, however, that an appetite stimulation resulting from the addition to an apparently inadequate diet of a constituent whose nutritional value is under investigation, is *prima facie* evidence of its indispensability.

In any case, we are still of the opinion that an experimental method, if based upon this merely plausible line of reasoning, cannot *demonstrate* anything, and the conclusions deduced from the results obtained, no matter how numerous or consistent they may be, should be stated with appropriate reservations.

[39] Karr, W. G., *J. Biol. Chem.,* 1920, xliv, 277.

[40] Hart, E. B., and Steenbock, H., *J. Biol. Chem.,* 1919, xxxviii, 269.

so that the possibility of uncontrolled experimental factors contributing to the body weight changes observed is much greater than in a properly controlled nitrogen balance study. The old belief that accurate nitrogen balance determinations on small experimental animals, such as the rat, are impossible has been shown to be incorrect.

THE SYNTHESIS OF AMINO ACIDS IN THE ANIMAL BODY

If amino acids normally formed as constituents of animal tissues and secretions need not be present in the diet, it is evident that the proof is at hand that these acids may be synthesized. However, the proof of the dispensabiliy of amino acids is more difficult to attain with the usual methods of investigation than a proof of their indispensability, since in the former case successful nutrition on diets *completely lacking* in the amino acid under investigation must be demonstrated, while in the latter case nutritive failure may be demonstrated on rations merely *deficient,* not totally lacking, in the indispensable amino acid.

The demonstration that a protein hydrolysate, however treated chemically, is totally deficient in a given amino acid is, with most amino acids, a difficult if not an impossible feat at the present writing. The best prospects for obtaining information as to which amino acids may be synthesized by the animal organism and are, therefore, dispensable constituents of the diet, would appear to relate to the feeding of synthetic mixtures of purified amino acids. Furthermore, in demonstrating adequate nutrition on an amino acid mixture known to be entirely free of a certain amino acid, it is essential to reduce all other sources of amino acids in the diet, such as the source of vitamin B, to a negligible minimum.

Abderhalden [32] has reported considerable success in the feeding of rations containing purified amino acids, but his experiments purporting to show that certain of the amino acids are dispensable, are marred by the inclusion in the diet of amounts of dried yeast (50 to 100 mgms. per day) not shown to be negligible as sources of the amino acids in question. The experiments indicate that leucine may replace norleucine and isoleucine, and that glycine, alanine, and hydroxy-glutamic acid may be dispensed with. Successful nutrition was not obtained on an amino acid mixture entirely devoid of six-carbon monoamino acids.

The attainment of successful nutrition on proteins in which glycine, hydroxy-glutamic acid, hydroxy-proline or other amino acids appear to be lacking cannot be urged as a demonstration that these amino acids are dispensable constituents of a complete diet, since the chemical methods available for detecting the presence of these amino acids are not suffi-

ciently sensitive to demonstrate their complete absence in a protein hydrolysate.

The most convincing evidence of the dispensability of certain amino acids as dietary constituents has been obtained by methods other than the simple feeding experiments hitherto considered. The dispensability of glycine has been demonstrated in experiments concerned with the detoxication of benzoic acid in certain animals. A review of much of this evidence is given by Shiple and Sherwin.[41] It may be briefly summarized as follows:

1. Hippuric acid may be formed in large quantities in mammals after the ingestion of benzoic acid. The amount of glycine thus conjugated and excreted is much larger than the amount present in the dietary protein. A man is able to convert a dose of 20 grams of benzoic acid quantitatively into hippuric acid.

2. The formation of hippuric acid following benzoic acid ingestion may occur on a nitrogen-free diet with little or no increase in the excretion of total urinary nitrogen.[41, 42] Under such conditions, however, a marked decrease in the urea excretion occurs, the percentage of urea nitrogen on the total urinary nitrogen attaining as low a value as 19 or even 12. Evidently nitrogenous metabolites normally excreted as urea are synthesized into glycine under the influence of benzoic acid poisoning. The excretion of creatinine nitrogen is not affected, indicating that detoxication of benzoic acid occurs without disturbing the endogenous catabolism of the tissues.

3. Swanson [43] has shown that after sodium benzoate ingestion, the concentration of urea in the blood does not increase, demonstrating that the decreased excretion of urea in the urine under these conditions is not the result of a retention of this compound.

While the synthesis of glycine in the animal organism thus appears to be established, the nature of the precursor or precursors from which the amino acid is formed has thus far eluded experimental enquiry. Perfusion experiments on the surviving liver, in which hippuric acid synthesis is known to occur, have yielded negative evidence with reference to the possibility of synthesis from other amino acids, hydroxy acids, or fatty acids.[44] Swanson [43] detected no appreciable change in the concentration of plasma amino acids subsequent to sodium benzoate ingestion in man, while the administration of other amino acids than glycine with the sodium benzoate apparently has no effect upon the rate

[41] Shiple, G. J., and Sherwin, C. P., *J. Amer. Chem. Soc.*, 1922, xliv, 618.
[42] McCollum, E. V., and Hoagland, D. R., *J. Biol. Chem.*, 1913-14, xvi, 321.
[43] Swanson, W. W., *J. Biol. Chem.*, 1925, lxii, 565.
[44] Friedmann, E., and Tachau, H., *Biochem. Z.*, 1911, xxxv, 88.

of hippuric acid synthesis, according to the experiments of Griffith and Lewis,[45] although the simultaneous ingestion of glycine itself markedly increased the rate of synthesis. The latter investigators in later experiments [46] have shown that the rate of synthesis of hippuric acid in rabbits was influenced by the simultaneous ingestion of protein in accordance with the glycine content of the protein, a result which was later confirmed by Csonka in experiments on the pig.[47] The possibility of the synthesis of glycine from the hydrolysis of the glycocholic acid of the bile, suggested by Zimmermann,[48] has received no support from the investigations of Rosenberg [49] or of Lewis.[50] Based upon the fact established by Dakin that phenylserine gives hippuric acid in the body while phenylalanine does not, Knoop [51] suggests that glycine originates by the oxidation of α-amino-β-hydroxy acids, such as serine or an intermediary metabolite of β-hydroxy-glutamic acid. However, the actual formation of glycine from such precursors has not been demonstrated.

While mammals detoxicate benzoic acid by conjugation with glycine, birds accomplish the same purpose by detoxication with ornithine, α-amino-valerianic acid, a compound differing from arginine only in the lack of the guanidine group in the delta position. This fact was first reported by Jaffé,[52] who called the conjugated compound (dibenzoyl-ornithine) ornithuric acid. Crowdle and Sherwin,[53] from experiments on benzoic acid conjugation in fowls, conclude that birds are able to synthesize this amino acid from nitrogenous metabolites that otherwise would be excreted as uric acid. However, the data can hardly be considered a demonstration of such a synthesis, since the total protein catabolism was markedly increased by benzoic acid administration. There is no assurance that this increased protein catabolism might not have furnished sufficient arginine for the detoxication of the benzoic acid administered.

A more successful demonstration of the ability of the animal organism to synthesize glutamic acid has been reported by Sherwin and his associates in experiments on the detoxication of phenylacetic acid. Thierfelder and Sherwin [54] have shown that glutamine is furnished by the human body for the detoxication of this acid, and it was later found by Sherwin, Wolf and Wolf [55] that the synthesis of 7.5 grams of glutamine

[45] Griffith, W. H., and Lewis, H. B., *J. Biol. Chem.*, 1923, lvii, 1.
[46] Griffith, W. H., and Lewis, H. B. *J. Biol. Chem.*, 1923, lvii, 697.
[47] Csonka, F. A., *J. Biol. Chem.*, 1924, lx, 545.
[48] Zimmermann, O., *Centr. inn. Med.*, 1901, xxii, 528.
[49] Rosenberg, S., *Centr. inn. Med.*, 1901, xxii. 696.
[50] Lewis, H. B., *J. Biol. Chem.*, 1921, xlvi, 73.
[51] Knoop, F., *Z. physiol. Chem.*, 1914, lxxxix, 151.
[52] Jaffé, M., *Ber. chem. Ges.*, 1887, x, 1925.
[53] Crowdle, J. H., and Sherwin, C. P., *J. Biol. Chem.*, 1923, lv, 365.
[54] Thierfelder, H., and Sherwin, C. P., *Ber. chem. Ges.*, 1914, xlvii, 2630; *Z. physiol. Chem.*, 1915, xciv, 1.
[55] Sherwin, C. P., Wolf, M., and Wolf., W., *J. Biol. Chem.*, 1918, xxxvii, 113.

was accomplished on a glutamic-acid-free diet without materially increasing the protein catabolism as indicated by the nitrogen and sulfur elimination in the urine. Finally Shiple and Sherwin [41] have demonstrated the synthesis of glutamine following phenylacetic acid ingestion on a diet practically protein-free. The synthesis apparently occurred at the expense of unknown nitrogenous metabolites that normally were excreted as urea. Both glycine and glutamic acid could be formed simultaneously after the simultaneous administration of benzoic and phenylacetic acid as readily as after the administration of either compound alone. This work of Sherwin's with reference to glutamic acid synthesis is in line with feeding experiments briefly reported by Hopkins [56] indicating the possibility of successful nutrition on amino acid mixtures from which both glutamic and aspartic acids were removed as completely as the Foreman method [57] permits.*

These positive indications of amino acid synthesis as steps in the detoxication of foreign compounds obtained for glycine and glutamic acid are to be compared with the negative results obtained by Muldoon, Shiple, and Sherwin [25] for cystine, an amino acid which the body is known to be unable to synthesize.

While it is thus well established that certain of the amino acids occurring in proteins may be synthesized in the animal organism, the method of synthesis normally followed is not clear. The probable production of keto or hydroxy acids in the deamination of amino acids naturally leads to the suspicion that these acids are the immediate precursors of the corresponding amino acids, a suspicion confirmed by the results of perfusion experiments with surviving livers by Embden and his associates. However, attempts to substitute for amino acids known to be indispensable in nutrition the corresponding hydroxy acids have not in all cases been attended with success in feeding experiments on rats. McGinty, Lewis, and Marvel [20] were unable to demonstrate a successful substitution of α-hydroxy-ε-aminocaproic acid for lysine, although more recently Cox and Rose [58] have reported a successful substitution of imidazole lactic acid for histidine.† Novelo, Harrow, and Sherwin [59] have

[56] Hopkins, F. G., *J. Chem. Soc.*, 1916, cix, 629.
[57] Foreman, F. W., *Biochem. J.*, 1915, viii, 463.
[58] Cox, G. J., and Rose, W. C., *Proc. Amer. Soc. Biol. Chem.*, Dec., 1925, 1; *J. Biol. Chem.*, 1926, lxviii, 781.

* Recent work by Bunney and Rose (*J. Biol. Chem.*, 1928, lxxvi, 521), also shows that rapid growth is possible on diets containing only very small amounts of glutamic and aspartic acids. Excellent growth was also secured upon rations practically devoid of arginine.

† Westerman and Rose (*J. Biol. Chem.*, 1927, lxxv, 533) were unable to secure evidence of a successful substitution for cystine of dithiodiglycolic acid or of β-dithiodipropionic acid in feeding experiments with rats. In later work (*ibid.*, 1928, lxxix, 413), negative results were also obtained with α-dihydroxy-β-dithiodipropionic acid. All of these disulfide acids proved to be readily oxidizable when administered orally or subcutaneously to rabbits (*ibid.*, 1928, lxxix, 423). However, if cysteine is the first metabolite of cystine, it may prove to be more profitable to work with cysteine derivatives rather than with cystine derivatives in experiments of this character.

[59] Novello, N. J., Harrow, B., and Sherwin, C. P., *Proc. Amer. Soc. Biol. Chem.*, Dec., 1925, 54. Harrow and Sherwin, *J. Biol. Chem.*, 1926, lxx, 683.

also reported the results of feeding experiments on rats with imidazole compounds, indicating a partial substitution of histidine by imidazole pyruvic acid and a more efficient substitution by imidazole lactic acid. It might be expected that the substitution of a hydroxy-acid for an amino acid would be as successful with lysine as with histidine; possibly the negative results obtained with lysine represent but another illustration of the uncertainties involved in the interpretation of simple feeding experiments in which the food consumption is uncontrolled. The positive results reported for histidine are more significant than the negative results reported for lysine. However, the justifiable conclusion that histidine and imidazole lactic acid are equivalent in metabolism is not readily reconciled with the experiments of Leiter [60] on the metabolism of imidazoles. On intravenous injection into a dog, histidine was almost completely metabolized, while imidazole lactic acid was excreted unchanged in the urine to the extent of 40 per cent, a greater loss than that resulting from the injection of the same amount (1 gram) of methyl imidazole, with which Cox and Rose obtained no favorable reaction as a substitute for histidine.

Although a high degree of probability has thus been established that, under certain conditions at least, the animal body is able to substitute an amino for a hydroxy group, only negative results have been obtained with reference to the ability to substitute an amino group for a hydrogen atom in a methyl or methylene group. Thus, McGinty, Lewis, and Marvel [20] obtained negative results in feeding experiments on the substitution of α-hydroxy-caproic, ε-hydroxy-caproic, and ε-amino-caproic acids for lysine, and Cox and Rose [58] obtained negative results on the substitution of 4-imidazole propionic (or 4-imidazole acrylic) acid for histidine. On the other hand, the presence of the β-alanyl group in carnosine (β-alanyl-histidine), a normal constituent of muscle tissue, indicates that such a substitution might be possible in animal metabolism.

THE METHOD OF PROTEIN SYNTHESIS

The preceding discussion illustrates the fact that it is much easier to demonstrate a given process in metabolism than it is to elucidate its *modus operandi,* and the question of protein synthesis *in vivo* is but another illustration. In the presence of a complete assortment of amino acids, their synthesis into protein is ordinarily naïvely dismissed as but an instance of the reversible phase of proteolysis. As a matter of fact, the recent work of Wasteneys and Borsook [61] has abundantly confirmed

[60] Leiter, L. *J. Biol. Chem.,* 1925, lxiv, 125.

[61] Wasteneys, H., and Borsook, H., *J. Biol. Chem.,* 1924, lxii, 15; 1925, lxii, 633, 675; lxiii, 563, 575. However, see Rona, P., and Chrometzka, Fr., *Biochem. Z.,* 1927, clxxxix, 249

the less convincing work of earlier investigators to the effect that the gastro-intestinal proteases, pepsin and trypsin, do synthesize as well as hydrolyze protein. Under suitable conditions of concentration, reaction, and temperature, the synthetic action may be emphasized to such an extent that 39 per cent of the nitrogen of the original digest will be found in the synthesized protein. However, the optimal conditions of synthesis cannot be normal in the body; *e.g.,* for pepsin a pH of 4.0 with inconsiderable synthesis at pH of 7.0, and for trypsin a pH of 5.7 with greatly reduced synthesis at a pH normal for the blood and tissues (7.4). Furthermore, at 38° C., synthesis fails in solutions of the products of proteolysis corresponding to 8 per cent or less of protein. It may be that the conditions of synthesis for the tissue proteases, which are known not to be either pepsin or trypsin,[62] will be found more consistent with conditions prevailing in the tissues. In all probability, as Bradley [62] points out, hypertrophy and growth require an abundant blood supply and a reaction sufficiently on the alkaline side of neutrality (pH 7.4 to 7.8) to insure the stability of the base-protein complexes of the tissues. The biological significance of the synthetic reactions investigated by Wasteneys and Borsook is obscure.

However, in spite of the possibility of the reversibility of proteolytic (autolytic) reactions under conditions normal to tissue life, it seems impossible to subscribe to the belief of Bradley, Sherman,[63] and others that protein synthesis in growth or hypertrophy is merely a reversal of the mechanism of protein hydrolysis in tissue autolysis. The active agents in the latter process are proteases whose action is apparently specific only to certain types of amino acid or peptide linkages. They are capable, according to Bradley's own investigations, of hydrolyzing a variety of proteins, just as are the proteases of digestion. On the other hand, the proteins of the tissues are of definite structure, determined largely if not entirely by hereditary factors, and quite unaffected by the proportions existing among the various amino acids in the intercellular fluids. It is inconceivable that catalysts destroying proteins regardless of their amino acid make-up and of their molecular pattern should synthesize such definite amino acid complexes of fixed pattern. The theory that protein synthesis in the tissues is but a reversal of autolysis thus assumes far more than the theory of reversible reactions. The problems of growth and hypertrophy are infinitely more complicated than the problems of autolysis and must involve factors not concerned with tissue destruction. The manner of protein synthesis in the body, therefore,

[62] Bradley, H. C., *Physiol. Rev.,* 1922, ii, 415.
[63] Sherman, H. C., *J. Biol. Chem.,* 1920, xli, 97.

must be placed among those biochemical problems still awaiting solution. It may represent a dehydration synthesis, as currently supposed, or it may represent a succession of reductions, polymerizations, and oxidations as Pauly [64] has suggested, or it may represent some other series of reactions, but in any case it represents a process rigidly directed by physiological factors unknown and unsuspected at the present writing.

AMINO ACIDS AS PRECURSORS OF NON-PROTEIN CONSTITUENTS OF ANIMAL TISSUES

If amino acids are available in anabolism only for the growth of new tissue, it would be expected that the "incomplete" proteins, zein and gelatin, could be utilized only for the production of energy or of glycogen or fat. In other words, if such were the case it would be expected that no nitrogen retention could occur after feeding such proteins; after a meal of gelatin or zein the cells would not find as complete an assortment of amino acids at their disposal as is required for the synthesis of new tissue protein or the replacement of old, and the only method of utilizing the amino acids absorbed from the blood would be to split off their nitrogen, which would be excreted in the urine, and to oxidize the non-nitrogenous residues or convert them into glycogen or fat.

McCollum,[65] however, has found that growing pigs may apparently retain as much as 80 per cent of the nitrogen of zein and 50 to 60 per cent of the nitrogen of gelatin, though no evidence was found of the formation of additional body tissue, even when zein was fed in great excess of the maintenance needs of the animal. From such results McCollum was led to the conclusion that "the processes of replacing nitrogen degraded in cellular metabolism are not of the same character as the processes of growth," and that the processes of cellular catabolism and repair do not represent a series of chemical changes involving the destruction and reconstruction of an entire protein molecule. This phase of protein anabolism will be discussed later in some detail in connection with the endogenous catabolism.

An explanation of the partial utilization of incomplete proteins in anabolism is afforded by the fact that other nitrogenous constituents of the tissues than proteins are in all probability dependent for their synthesis upon certain specific amino acids. The relation of tyrosine or phenylalanine to epinephrine, of tyrosine to thyroxin, of histidine to carnosine, of arginine to creatine, and of cystine to glutathione and to

[64] Pauly, H., *Z. physiol. Chem.*, 1917, xcix, 161.
[65] McCollum, E. V., *Wisc. Agri. Exp. Sta. Res. Bull.* No. 21, 1912, p. 58.

taurine may be mentioned in this connection. The nature of these relationships and of the transformations involved will be taken up in more detail, as available information permits, in succeeding sections devoted to the distinctive features in the metabolism of individual amino acids.

CHAPTER VI

GENERAL AMINO ACID METABOLISM. II. CATABOLISM AND UTILIZATION AS SOURCES OF ENERGY.

The peculiar function of protein in the body that, in practical nutrition, cannot be served by any other nutrient, is to provide structural units (amino acids) for the synthesis of the nitrogenous constituents (protein and non-protein in character) of the tissues and secretions of the body. However, the amino acids coming to the tissues from the alimentary canal are readily oxidizable, except for their nitrogen-containing radicals, and may, therefore, at any time be drawn into the energy exchanges of the total tissues. The extent to which they are thus utilized as sources of energy will depend largely upon the proportionate amounts and the character of the non-nitrogenous nutrients coming to the tissues simultaneously with the amino acids. Also, if protein is being consumed in excess of the needs of the body for nitrogenous structural units, the excess consumed will be oxidized directly or, if the energy requirements of the body are more than satisfied by the food consumed, the excess may be stored as glycogen or fat. Therefore, the main factors determining what proportion of the incoming amino acids will be diverted from synthetic reactions to deamination may be enumerated as follows:

a. The excess of amino acids absorbed from the gastro-intestinal tract above the maximum requirement for amino acids by all of the tissues in the course of their normal functioning.

b. The adaptability of the assortment of amino acids resulting from digestion in satisfying the requirements of the tissues. The more suitable the assortment of amino acids for the purposes of synthesis into tissue proteins or other nitrogenous constituents, the smaller the proportion of the incoming amino acids that will necessarily be deaminized. However, in the presence of an excess of amino acids (see *a*) or of a deficiency of non-protein energy-yielding material (see *c* and *d*) in the food consumed, the effect of this factor will be discounted or even, in extreme cases, be entirely obliterated.

c. The proportion of amino acids in the digestible nutrients consumed.[1] The smaller this proportion, the greater the "protein-sparing"

[1] Mitchell, H. H., *J. Biol. Chem.*, 1924, lviii, 905.

effect of the non-nitrogenous nutrients, and the smaller the proportion of the incoming amino acids that will be drawn into the energy metabolism of the body, deaminized and oxidized.

d. The ratio between the available food energy consumed and the total energy requirements of the body. As this ratio diminishes from 1, the proportion of the incoming amino acids that will be diverted to catabolism and energy production will increase.

DEAMINATION

The first step in the oxidation of an amino acid or in its conversion into glucose or fat is the removal of its amino group. The animal body possesses only a very limited capacity for the oxidation of nitrogen, confined apparently to the oxidation of the nitrite to the nitrate radical.[2] Hence the purpose of deamination may be considered as the removal of an unoxidizable fragment of the amino acid. While it is possible to conceive of deamination as a reduction according to the following equation

$$\begin{matrix} \mathrm{R.CH.COOH} \\ | \\ \mathrm{NH_2} \end{matrix} + 2\mathrm{H} = \mathrm{R.CH_2.COOH} + \mathrm{NH_3},$$

in all probability it actually occurs in animal tissues as the result of either hydrolysis or oxidation—

$$\begin{matrix} \mathrm{R.CH.COOH} \\ | \\ \mathrm{NH_2} \end{matrix} + \mathrm{H_2O} = \begin{matrix} \mathrm{R.CH.COOH} \\ | \\ \mathrm{OH} \end{matrix} + \mathrm{NH_3}$$

$$2\begin{matrix} \mathrm{R.CH.COOH} \\ | \\ \mathrm{NH_2} \end{matrix} + \mathrm{O_2} = 2\mathrm{R.CO.COOH} + 2\mathrm{NH_3}$$

Of these two most probable methods of deamination, the latter, or oxidative deamination, is presumed, on the basis of indirect evidence, to represent the usual method. The reason for concluding that oxidative deamination, rather than hydrolytic deamination, is the usual method have been presented in a very complete fashion by Dakin.[3] They may be summarized briefly as follows:

(*a*) The α-ketonic acids are, in general, more readily oxidizable than the α-hydroxy acids, as revealed by the results of subcutaneous injection [4] and by perfusion experiments on the surviving liver.[5]

(*b*) In general, the fate in the body of α-amino and α-ketonic acids is identical, whereas the α-hydroxy acids may behave differently. For

[2] Mitchell, H. H., Shonle, H. A., and Grindley, H. S., *J. Biol. Chem.*, 1916, xxiv, 461.
[3] Dakin, H. D., "Oxidations and Reductions in the Animal Body," London, 1922, 2nd ed. Pp. 64 to 71.
[4] Suwa, A., *Z. physiol. Chem.*, 1911, lxxii, 113.
[5] Kotake, Y., *Z. physiol. Chem.*, 1910, lxix, 409.

example, Neubauer and Gross [6] found that *p*-hydroxyphenyl-lactic acid does not readily yield acetoacetic acid on perfusion through the surviving liver, while the corresponding *α*-amino and *α*-ketonic acids (tyrosine and *p*-hydroxyphenyl-pyruvic acid) yield acetoacetic acid freely. Similarly Friedmann and Maase [7] observed that *p*-chlorphenyl-alanine and *p*-chlorphenylpyruvic acid were converted into *p*-chlorphen-aceturic acid

$$\begin{array}{l} \mathrm{Cl.C_6H_4.CH_2.CO} \\ \qquad\qquad\quad | \\ \qquad\qquad\quad \mathrm{NH} \\ \qquad\qquad\quad | \\ \qquad\qquad\quad \mathrm{CH_2.COOH} \end{array}$$

on oxidation in the body, while *p*-chlorphenyl-lactic acid was not.

Further evidence of the same significance has been obtained from human subjects suffering from the peculiar metabolic disturbance known as "alcaptonuria." Persons in this condition are not able to oxidize completely the aromatic amino acids phenylalanine and tyrosine. The oxidation is carried to 2, 5-dihydroxyphenylacetic acid (homogentisic acid), which is excreted in the urine. Neubauer [8] determined that both tyrosine and *p*-hydroxyphenyl-pyruvic acid when administered to alcaptonurics by mouth yield homogentisic acid, while *p*-hydroxyphenyl-lactic acid does not.

On the basis of such evidence as that just reviewed, Dakin concludes that *α*-ketonic acids are obligate products of the direct oxidation of amino acids; in other words, that hydrolytic deamination does not occur in the animal body. It is true that *α*-hydroxy acids may be produced from *α*-amino acids after excessive feeding of the latter [9] or by their perfusion through the surviving liver,[10] but Dakin considers their production as indirect, by reduction of *α*-ketonic acids or by other means. Knoop and Kertess [11] argue for the same conclusion from experiments on a dog to which was administered *dl-p*-phenyl-*α*-amino-butyric acid (*per os*) and, in another period, the corresponding *α*-ketonic acid (subcutaneous injection) as the sodium salt. In both periods, small amounts of the *d-α*-hydroxy acid were recovered in the urine among other products. The fact that the same hydroxy acid was obtained from the ketonic acid as from the amino acid, and in larger amount, was taken to indicate that the hydroxy acid was formed from the amino acid by way of the ketonic acid. However, the larger amount of the hydroxy

[6] Neubauer, O., and Gross, W., *Z. physiol. Chem.*, 1910, lxvii, 219; see also, Schmitz, E., *Biochem. Z.*, 1910, xxviii, 117.
[7] Friedmann, E., and Maase, C., *Biochem. Z.*, 1910, xxvii, 97.
[8] Neubauer, O., *Deut. Arch. klin. Med.*, 1909, xcv, 211.
[9] Neuberg, C., and Langstein, L., *Arch. Physiol.*, 1903, Suppl. Bd., 514. Kotake, Y., *Z. physiol. Chem.*, 1910, lxv, 397. Mayer, P., *Z. physiol. Chem.*, 1904, xlii, 59.
[10] Embden, G., and Schmitz, E., *Biochem. Z.*, 1910, xxix, 423.
[11] Knoop, F., and Kertess, E., *Z. physiol. Chem.*, 1911, lxxi, 252.

acid obtained from the ketonic acid than from the amino acid may be related to the difference in the method of administration. The argument also assumes tacitly that the asymmetric reduction of an α-ketonic acid in the animal body is not possible, contrary to the conclusions of Kotake and others.

However, Abderhalden argues [12] that no general statement can be made as to the method of formation of α-hydroxy acids. While in some cases they appear to be products only of the reduction of α-ketonic acids, in other cases there is no reason to doubt their direct formation from α-amino acids, while in other cases good evidence exists for a direct formation. Thus, *d*- and *l*-phenyl-lactic acid like phenylalanine are readily converted into acetoacetic acid in the surviving liver,[13] while phenyl-pyruvic acid is not,[14] and in the alcaptonuric, phenyl-lactic acid, like phenylalanine, yields homogentisic acid, according to Neubauer and Falta,[15] in contrast to the failure of hydroxy-phenyl-lactic acid, to which reference has already been made. In proof of a direct hydrolytic deamination, Abderhalden cites, on the one hand, the experience of Kotake [9] demonstrating the appearance in the urine of *l*-*p*-hydroxy-phenyl-lactic acid after an abundant feeding of tyrosine (to dogs) and, on the other hand, the observation of Suwa [4] (on men) that ingestion of *p*-hydroxy-phenyl-pyruvic acid leads to the excretion of *d*-*p*-hydroxy-phenyl-lactic acid, representing an asymmetric reduction. The argument is further strengthened by the later work of Kotake and Okagawa,[16] demonstrating with rabbits that while both *l*-tyrosine and *d,l*-tyrosine are converted to about the same extent into *p*-hydroxy-phenyl-pyruvic acid, only *l*-tyrosine gives rise to any but inappreciable amounts of *l*-hydroxy-phenyl-lactic acid, and by the experiments of Kotake, Masai and Mori,[17] who found that in rabbits injected with carmine, with the purpose of diminishing the oxidative activity of the reticulo-endothelial tissues, administration of phenylalanine and tyrosine *per os* did not lead to the excretion of α-ketonic acids in the urine, although in one case a relatively large amount of *l*-hydroxy-phenyl-lactic acid was obtained from *l*-tyrosine. Similarly [18] in surviving livers vitally stained by this method, the ability to convert phenylalanine to tyrosine is increased rather than impaired, while the formation of acetoacetic acid was not greatly affected. Apparently the inhibition of oxidative deamination does not interfere with these transformations.

[12] Abderhalden, E., "Lehrbuch der Physiologischen Chemie," 5 Auflage, 1 Teil, Berlin, 1923.
[13] Mori, Y., *Z. physiol. Chem.*, 1922, cxxii, 225.
[14] Embden, G., and Baldes, K., *Biochem. Z.*, 1913, lv, 301.
[15] Neubauer, O., and Falta, W., *Z. physiol. Chem.*, 1904, xlii, 81.
[16] Kotake, Y., and Okagawa, M., *Z. physiol. Chem.*, 1922, cxxii, 201.
[17] Kotake, Y., Masai, Y., and Mori, Y., *Z. physiol. Chem.*, 1922, cxxii, 211.
[18] Kotake, Y., Masai, Y., and Mori, Y., *ibid.*, p. 220.

The work of Ringer on phlorhizinized dogs might also be cited in this connection, particularly in view of the fact that all of the evidence thus far cited refers to the aromatic amino acids. Ringer showed that, while alanine and lactic acid never fail to yield large quantities of extra glucose in the urine of such subjects rendered diabetic by phlorhizin,[19] pyruvic acid at times yields very small quantities.[20] The results of these experiments indicate that alanine and lactic acid are quantitatively interchangeable in the diabetic organism, while pyruvic acid is not so intimately related to alanine.

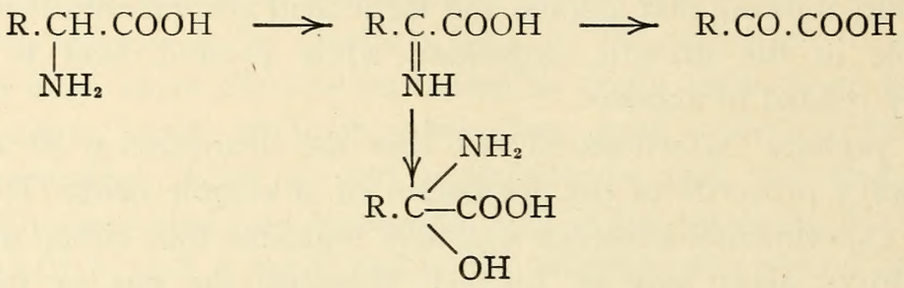
It is perhaps hazardous to say that the deamination of α-amino acids always proceeds to the formation of α-ketonic acids. The fragmentary experimental evidence available indicates that either α-ketonic or α-hydroxy acids may be formed. Whether the one or the other product results from deamination may depend upon the particular amino acid under consideration, or upon the species of animal to which the amino acid is administered, or upon the condition of the animal or of the administration. As Dakin amply demonstrates, the relations between α-amino, α-ketonic, and α-hydroxy acids are extremely close, and the interactions, whatever they may be, are readily reversible.

Of particular interest is the demonstration by Knoop [21] that the administration to a dog *per os* of either γ-phenyl-α-ketobutyric acid or γ-phenyl-α-hydroxy-butyric acid resulted in the excretion in the urine of the corresponding α-amino acid. Embden and associates have applied their method of liver perfusion to the same problem, and have been able to demonstrate the production of alanine, leucine, phenylalanine, and tyrosine from the ammonium salts of the corresponding α-ketonic acids.[22] Similarly, α-amino-n-butyric acid and α-amino-n-caproic acid were obtained in the same fashion.[23] The production of alanine from ammonium lactate [24] and from glycogen-rich, in contrast to glycogen-poor, livers [25] has also been accomplished. These synthetic reactions leading to the production of amino acids from non-nitrogenous compounds are of fundamental biological significance in demonstrating the possible reversibility of the deamination reactions. However, their significance may be greatly exaggerated when they are taken to mean that under normal conditions amino acids are synthesized after this fashion.

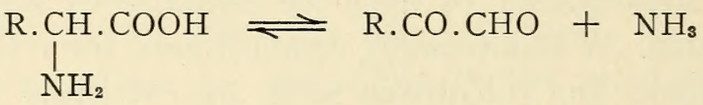
Whether α-hydroxy or α-ketonic acids result from the deamination

[19] Ringer, A. I., and Lusk, G., *Z. physiol. Chem.*, 1910, lxvi, 106. Mandel, J. A., and Lusk, G., *Amer. J. Physiol.*, 1906, xvi, 129.
[20] Ringer, A. I., *J. Biol. Chem.*, 1913, xv, 145.
[21] Knoop, F., *Z. physiol. Chem.*, 1910, lxvii, 489.
[22] Embden, G., and Schmitz, E., *Biochem. Z.*, 1910, xxix, 423; *ibid.*, 1912, xxxviii, 393.
[23] Kondo, K., *Biochem. Z.*, 1912, xxxviii, 407.
[24] Embden, G., and Kraus, F., *Biochem. Z.*, 1912, xlv, 1.
[25] Fellner, H., *Biochem. Z.*, 1912, xxxviii, 414.

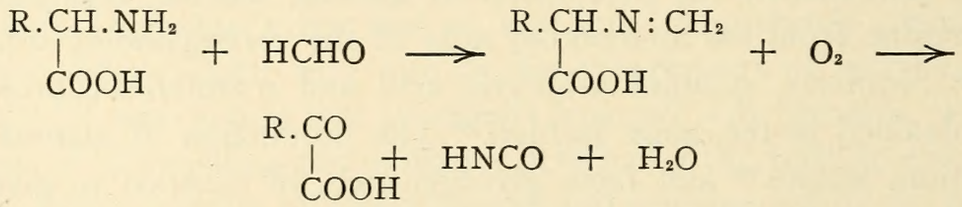
of α-amino acids *in vivo*, the reaction cannot be considered as proceeding in one step. The nature of the intermediate products formed is, however, a matter of conjecture. Without attempting a discussion of this interesting but experimentally inaccessible problem, reference may be made to the suggestion of Knoop [26] of the intermediate formation of an α-imino acid or its hydrate—



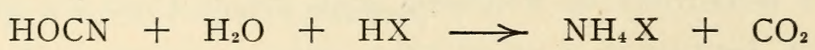
to the interesting suggestion of Dakin and Dudley [27] that the intermediate products are α-ketonic aldehydes (glyoxals)—



and to the more recent suggestion of Fearon and Montgomery,[28] based upon *in vitro* oxidation experiments only, that the formation of cyanic acid, rather than of ammonia, is the direct result of deamination. Cyanic acid formation in the amino-acid-oxidizing mixtures was favored greatly by the presence of glucose or formaldehyde, so the reaction is pictured as follows:



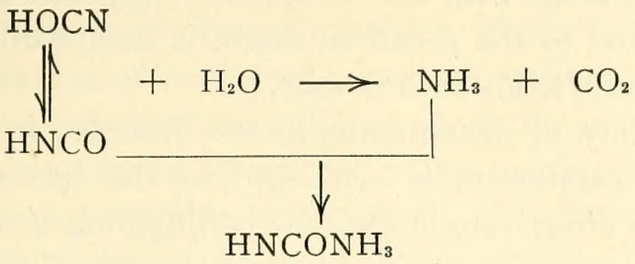
This suggested type of deamination overcomes the difficulty of assuming an ammonia formation by oxidation in alkaline solution, since, as emphasized by the recent work of Fosse, ammonia itself is readily oxidized under such conditions in the presence of carbon compounds. On the other hand, cyanic acid is stable to oxidizing acids. In the presence of acids, cyanic acid is completely hydrolyzed to ammonia:



In aqueous solution alone, cyanic acid is in part hydrolyzed to ammonia

[26] Knoop, F., *Z. physiol. Chem.*, 1910, lxvii, 489; 1925, cxlviii, 294.
[27] Dakin, H. D., and Dudley, H. W., *J. Biol. Chem.*, 1913, xiv, 555; xv, 127; 1914, xviii, 29.
[28] Fearon, W. R., and Montgomery, E. G., *Biochem. J.*, 1924, xviii, 576. *See also*, Fearon, W. R., *Physiol. Revs.*, 1926, vi, 399.

and carbon dioxide, and in part converted into urea by union with ammonia



In the presence of an excess of ammonia, the conversion to urea is complete. The subsequent detection by Montgomery [29] of cyanic acid in the blood of cats after protein feeding contributes some support to this theory of deamination.

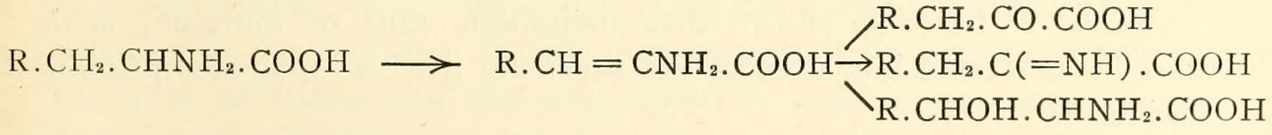
The theory of Dakin and Dudley is particularly attractive, not only because of its experimental justification, but also because the glyoxals thus formed have been shown to be convertible *in vivo* either into α-hydroxy acids by hydration—

$$R.CO.CHO + H_2O \rightleftharpoons R.CHOH.COOH$$

or into α-ketonic acids by oxidation—

$$2\,R.CO.CHO + O_2 = 2\,R.CO.COOH$$

The former transformation is a property of many tissues and is evidently due to the catalytic action of an enzyme that has been called glyoxalase. The latter transformation has been demonstrated by perfusing a surviving liver with phenyl glyoxal and isolating phenyl glyoxylic acid from the perfusate. The theory also permits a better understanding of the relations between amino acid and carbohydrate metabolism, because of the existence of a common metabolite, methyl glyoxal, and particularly of the conversion of various amino acids into glucose in the diabetic organisms. More recently Dakin [30] has offered still another suggestion, *i.e.*, that α-amino acids may undergo the first step in β-oxidation of the type well established by Knoop for normal fatty acids, with the production of an α-amino-α,β-unsaturated acid, the immediate possible transformations of which are as depicted below—



This suggestion would thus account for the production of α-imino acids in accordance with Knoop's ideas. The experimental confirmation of it is, however, admittedly weak, consisting of the demonstration that

[29] Montgomery, E. G., *Biochem. J.*, 1925, xix, 71.
[30] Dakin, H. D., *J. Biol. Chem.*, 1926, lxvii, 341.

α-amino-β-trimethyl propionic acid, in which, unlike all the biologically important amino acids with the exception of glycine, there is no hydrogen atom attached to the β-carbon atom, is combustible with difficulty on subcutaneous injection into rabbits.

The importance of deamination as the first step in the utilization of amino acids in catabolism is evidenced by the fact that oxidation of an amino acid is effectively blocked by conjugating it with various other compounds through its amino group. Magnus-Levy [31] found that benzoylated amino acids were thus protected; Shiple and Sherwin [32] reported the same to be true of phenylacetylated amino acids; while Lewis and Root [33] were able to protect cystine from oxidation by conjugating it with phenylisocyanate, giving a uramino compound. Friedmann [34] noted that when the amino acids are simply monomethylated, the lower members of the series were excreted unchanged to the extent of one-third, while the higher members of the series were excreted almost quantitatively.

THE FORMATION OF UREA

As early as 1882, Schroeder [35] demonstrated the ability of the liver to form urea from the ammonium salts of organic acids, actually isolating urea nitrate from the perfusion fluid. This function of the liver has been so clearly demonstrated that it is a well-established fact. In the intact animal body it is evident from analyses of the blood in the portal vein and in the hepatic vein or in the vena cava that one important function of the liver is to remove the excess of ammonia coming to it, mainly from the large intestine; [36] in all probability, the absorbed ammonia is converted into urea. This is only one of the liver's many protective functions against the inflow of toxic products, mainly of bacterial action upon proteins, from the gastro-intestinal tract. The change of ammonium carbonate, remaining from the oxidation of the organic acid, to urea is presumed to be a dehydration, passing through the ammonium carbamate stage:

$$NH_4.O.CO.O.NH_4 \longrightarrow NH_4.O.CO.NH_2 \longrightarrow NH_2.CO.NH_2$$

Löffler [37] has also shown that ammonium salts of inorganic acids may be converted into urea by the surviving liver, as may also primary amines.

The ability of the liver to form urea from amino acids has been

[31] Magnus-Levy, A., *Biochem. Z.*, 1907, vi, 541.
[32] Shiple, G., and Sherwin, C. P., *J. Biol. Chem.*, 1922, l, 671.
[33] Lewis, H. B., and Root, L. E., *J. Biol. Chem.*, 1922, l, 303.
[34] Friedmann, E., *Beitr. chem. Physiol. Path.*, 1908, xi, 158.
[35] Schroeder, W. v., *Arch. exp. Path. Pharm.*, 1882, xv, 364.
[36] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 161.
[37] Löffler, W., *Biochem. Z.*, 1918, lxxxv, 230.

investigated by many different methods with such contradictory results that it seems impossible to reconcile the recorded experiments or, in most cases, to explain their divergent results. Much of the confusion is probably due to the use of inadequate chemical methods, to the selection of unfavorable experimental conditions, or to the inadequate control of experimental procedures. A review of the earlier literature, therefore, hardly seems a profitable undertaking. Therefore, discussion will be confined largely to those published investigations of the last fifteen years that seem the most effective in yielding unequivocal experimental evidence.

Up to 1913, the most direct evidence obtained from perfusion experiments relative to the ability of the liver to deaminize amino acids was obtained by Salaskin.[38] However, the experiments of Salaskin have been criticized by Fiske and Karsner [39] mainly on two grounds. The most pronounced indications of urea formation were obtained in the last half of the perfusion periods, which lasted for 3 to 4 hours, when the liver was undoubtedly in a less normal functional condition. Hence the results might have been complicated by autolysis or bacterial activity. Furthermore, the Schöndorff method used for the determination of urea gives results on blood comparable (when expressed as nitrogen) to the total non-protein nitrogen by present day methods, and two or three times the actual urea values. Fiske and Karsner, in attempting to confirm Salaskin's experiments, perfused the surviving livers of cats and of rabbits with homologous defibrinated blood containing either ammonium carbonate or glycine. They found that the surviving liver is capable of removing ammonium carbonate from the perfusing fluid and of converting it at least partially into urea. However, in the glycine experiments no increase in the urea content of the perfusing fluid could be demonstrated. In these experiments the analytical methods of Folin were used.

In 1915, Jansen [40] again attempted a confirmation of Salaskin's experiments. In commenting upon the work of Fiske and Karsner, Jansen questioned the efficiency of the perfusion method used because of no provision for the oxygenation of the perfusing fluid. He calls attention to the fact that, in later experiments [41] on surviving livers in which the perfusion fluid was arterialized by shaking with pure oxygen, a much more complete removal of ammonia could be effected than in the previous experiments. An attempt to demonstrate a deamination of

[38] Salaskin, S., *Z. physiol. Chem.*, 1898, xxv, 128.
[39] Fiske, C. H., and Karsner, H. T., *J. Biol. Chem.*, 1913, xvi, 399.
[40] Jansen, B. C. P., *J. Biol. Chem.*, 1915, xxi, 557. Also, *Nederlandsch. Tijdschrift Geneeskunde*, 1915, issue of Nov. 20.
[41] Fiske, C. H., and Karsner, H. T., *Biol. Chem.*, 1914, xviii, 381.

amino acids was, unfortunately, not made in these later experiments, nor were any urea determinations made, but the possibility exists, nevertheless, that different results might have been obtained in the first experiments with glycine if arterialization of the perfusion fluid had been accomplished. In Jansen's experiments, therefore, such a procedure was adopted and marked increases in the urea content of the perfusates were demonstrated when ammonium carbonate, glycine, alanine, or ereptone were present. However, with no amino acid additions, a considerable and variable accumulation of urea in the perfusing fluid was obtained in two experiments, a result later obtained by Löffler.[37] This accumulation was such as to render the increases noted in several of the amino acid experiments of dubious significance as criteria of deamination. The author admits as much for the leucine experiments, and, with equal justification, some of the experiments with the other additions must be considered inconclusive. Other experiments, however, resulted in urea accumulations so much greater than those noted in the two control experiments as to leave little room for doubt that the surviving liver does possess the capacity to deaminize amino acids and to produce urea from the products of deamination.

The results of Jansen were later substantially confirmed by the perfusion experiments of Löffler,[42] who obtained positive results for a series of amino acids including glycine, leucine, and serine, but negative results for tyrosine and cystine. The ability of the surviving liver to form urea from amino acids has thus been amply demonstrated, while no demonstration of such a function in other surviving tissues has thus far been submitted.

A large amount of work has been done upon the capacity of autolyzing tissues to produce ammonia or urea from their own substance or from added amino acids. Much of this work is questionable, however, because of the difficulty of carrying out such experiments under aseptic conditions, while the significance of the ammonia production obtained in experiments conducted in the presence of antiseptics is ambiguous, since it may result either from deamination or deamidation. Also, the difficulties in obtaining significant ammonia determinations in fresh biological material have only recently been realized and apparently overcome. The production of urea in autolyzing tissue is a much more significant finding than a production of ammonia.

The production of urea in liver autolysis, after much contradictory earlier work, was first demonstrated by Fosse and Rouchelman[43] in

[42] Löffler, W., *Biochem. Z.*, 1916, lxxvi, 55.
[43] Fosse, R., and Rouchelman, N., *Compt. rend.*, 1921, clxxii, 771.

1921, using the xanthydrol method of urea determination. In these experiments, a six-fold increase of urea in autolyzing liver tissue was observed. The formation of urea was entirely inhibited by preliminary boiling of the liver tissue. Fosse [44] believes that the immediate precursor of urea is cyanic acid. McCance [45] obtained urea formation not only in autolyzing liver (ox, sheep, pig, and rabbit), but also in autolyzing spleen (ox, cat), and to a less extent in autolyzing kidney (ox, sheep, pig). No urea production was noted in mammalian pancreas, lung, skeletal muscle, or heart muscle. Avian liver (hen) also gave negative results. Ammonia production was demonstrable in all autolyses. In this connection it is interesting to note that arginase, an enzyme capable of producing urea directly from arginine, has been found in mammalian (but not in avian) livers, and in kidneys to a less extent. No arginase has been found in spleen tissue. Hence, while some (or possibly all) of the urea produced in autolyzing liver and kidney tissue may result from the action of arginase, some other explanation must account for the urea production from spleen. However, it is difficult to believe that the rapid production of urea in autolyzing liver can be entirely accounted for by the action of arginase upon the minimal amounts of free arginine present in fresh liver or upon the arginine liberated by proteolysis. The recent experiments reported by Myers, Ringer and Benson [46] serve to confirm this view. Here a tenfold increase in urea was demonstrated in liver autolyses, although the free amino nitrogen increased only to an insignificant extent. In confirmation of earlier work by Hoagland and Mansfield,[47] no urea formation could be demonstrated in autolyzing muscle tissue.

It is interesting to observe that McCance [45, 48] has shown that urea formation in autolyzing tissue is inhibited by the presence of oxygen. McCance has taken this to mean that urea cannot have its origin from the oxidation of amino acids, or of glucose in the presence of ammonia.[49]

Recent experiments by Luck [50] on ammonia and urea formation resulting from the incubation of tissue suspensions with various amino acid mixtures in the presence of an antiseptic (toluene) have a significance similar to that of autolysis experiments. A large number of different tissues (nineteen) were thus tested. None of them, including liver and kidney tissue, gave any distinct indication of ammonia production from hydrolyzed gelatin, an amide-free substrate. Several

[44] Fosse, R., *Bull. Soc. chim. Biol.*, 1920, ii, 4.
[45] McCance, R. A., *Biochem. J.*, 1924, xviii, 486.
[46] Myers, V. C., Ringer, M., and Benson O. O., *Proc. Soc. Exp. Biol. Med.*, 1926, xxiii, 474.
[47] Hoagland, R., and Mansfield, C. M., *J. Biol. Chem.*, 1917, xxxi, 487.
[48] McCance, R. A., *Biochem. J.*, 1925, xix, 134.
[49] Fosse, R., *Compt. rend.*, 1912, cliv, 1448; *ibid.*, 1919, clxviii, 908.
[50] Luck, J. M., *Biochem. J.*, 1924, xviii, 814.

tissues (liver and kidney, and less distinctly gastric and intestinal mucosa) produced ammonia from amide-containing substrates. Hence, this ammonia production was ascribed to deamidation, and the conclusion was deduced that deamination was not accomplished under the conditions of these experiments by any of the numerous tissues tested. A production of urea was observed only in the case of liver tissue, intestinal mucosa, and kidney tissue. In the course of these investigations, the interesting observation was made that the gastric mucosa of many animals contains a true urease, capable of hydrolyzing urea to ammonia.[50, 51]

In 1919, Hammett [52] demonstrated variable increases in the urea content of autolyzing human placentas, ranging from practically 0 to 228 per cent. This finding lends further importance to the placenta as an intermediary organ between mother and fetus for the carrying on of necessary metabolic processes undeveloped by the fetus.

The investigations thus far reviewed indicate that muscle tissue is incapable of urea formation and that of the glandular tissues, only a few possess this function, while of these few the liver is preëminent, not only because of the relative intensity of the reaction exhibited by liver tissue, but also because of the relatively enormous size of the organ itself. It is natural, therefore, that the opinion should have gained currency that the liver is the chief site of urea formation, if not of deamination. However, attempts to confirm this belief have not been wholly successful. Determinations of the relative urea content of the blood and tissues of animals, while occasionally indicating a distinctly higher content in the liver than in the muscles,[53] have not added appreciably to the conclusion that the liver is mainly responsible for the formation of urea. Indeed, it is doubtful whether such an experimental procedure is capable of throwing much light upon the question, in view of the rapid diffusibility of urea into all tissues of the body demonstrated by Marshall and Davis.[54] Similarly, methods concerned with the relative urea content of the arterial and venous blood supply to different organs or regions of the body seem impotent in this regard. Folin and Denis [55] cite the fact that they have been unable to demonstrate a higher content of urea in the blood from the hepatic vein than in the blood from the iliac artery taken at almost the same time during a period of active urea formation as evidence that the liver possesses no special rôle in this respect. However, their experimental results also show no difference in

[51] Luck, J. M., *Biochem. J.*, 1925, xix, 357.
[52] Hammett, F. S., *J. Biol. Chem.*, 1919, xxxvii, 105.
[53] Herter, C. A., *Johns Hopkins Hosp. Rpt.*, 1900, ix, 69.
[54] Marshall, E. K., Jr., and Davis, D. M., *J. Biol. Chem.*, 1914, xviii, 53.
[55] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xii, 141.

urea content between the blood of the iliac artery and that of the iliac vein taken almost simultaneously. Folin and Denis conclude that "the negative results, so far as any localized urea formation is concerned, is almost satisfactory proof that there is none, for if there were one central focus from which all or nearly all of the urea originated we could scarcely have failed to find it." It is, perhaps, a permissible conclusion, however, that an experimental method that fails to demonstrate urea formation in *any* of the tissues of the body at a time when urea is rapidly being formed, as demonstrated by its accumulation in the systemic blood, is not a method possessing sufficient precision to throw any light at all upon the question whether urea formation is a generalized tissue function or one confined to a particular organ.

Three years after the publication of the results of Folin and Denis, just reviewed, Taylor and Lewis [56] also attacked the problem of the predominance of the liver in urea formation by methods of blood analysis. In four dogs from which the whole alimentary tracts had been removed, they were able to demonstrate consistently a greater urea + ammonia content of the hepatic venous blood than of the blood in the posterior vena cava. However, by a peculiar argument based upon the assumption that creatinine, not urea, is the chief nitrogenous product of the muscular system, they conclude that "the slight excess of urea N present in hepatic blood over the urea N of peripheral blood certainly does not speak for any special predominance of the liver in the total function of formation of urea. The figures, instead, speak for the view that the formation of urea is the function of all tissues." The excess of urea in the hepatic blood amounted, in the average, to approximately 20 per cent at a time when no very active catabolism of dietary protein could have occurred, and when the formation of urea by the liver from the ammonia of the portal blood was absolutely prevented. The conclusion, therefore, that this experimental result has no positive significance with reference to the object of the experiment seems equivalent to the admission that the whole method of attack was incompetent to serve the purpose for which it was devised.

Other methods of deciding the relative importance of the liver in urea formation involve its partial or complete removal from the circulation, its partial or complete destruction by specific poisons or by disease, and its partial or complete removal from the body. In general, these methods, as reviewed in a recent publication,[57] have indicated a marked disturbance in urea formation revealed by blood or urine

[56] Taylor, A. E., and Lewis, H. B., *J. Biol. Chem.*, 1915, xxii, 77.
[57] Bollman, J. L., Mann, F. C., and Magath, T. B., *Amer. J. Physiol.*, 1924, lxix, 371; 1926, lxxviii, 258.

analysis. Particular reference may be made to the work of Stadie and Van Slyke [58] on protein metabolism in acute yellow atrophy of the liver. With reference to those experiments in which an attempt was made completely to remove the liver from the body or from the blood circulation, it should be mentioned, on the one hand, that the leaving of even a small residue of liver tissue around the vena cava may defeat the purpose of the experiment and complicate to an unknown extent the interpretation of the results obtained,[59] while, on the other hand, the complete isolation of the liver cannot be considered to have been accomplished merely by ligating the gross arteries and veins leading to it. Rich [60] has shown that in dogs some blood circulation through the liver by way of the diaphragmatic vessels may be demonstrated after the main blood supply to the organ has been occluded by ligatures. This blood supply can be cut off only by ligaturing the vena cava between the liver and the diaphragm, since these vessels run along the surface of the vena cava.

The most important evidence that has thus far been adduced in support of the belief that urea formation is a generalized function of all tissues in the body rather than a function characteristic of liver tissue only is afforded by the experimental data of Fiske and Sumner [61] reported from Folin's laboratory in 1914. The experiments were performed upon cats in which the isolation of the liver was attempted by ligation of the celiac artery, the superior and inferior mesenteric arteries, the hepatic artery, the portal vein, and the bile duct. The renal vessels were also ligated. Control animals had only the kidneys isolated. With no injection of amino acids, small and comparable increases were noted in the urea content of the blood of both control and "operated" animals in the course of 2 to 3 hours, although no increases in the urea content of muscle could be demonstrated. Two series of experiments were performed in which amino acid solutions (either glycine or alanine) were injected intravenously. The first experiments lasted 3 hours, and the "operated" animals were moribund even before the amino acid injections were complete. In this series of experiments increases in the urea content of the blood were noted in all "operated" animals, though they were less marked than those observed in the controls. The urea content of the muscles, on the other hand, increased markedly in the controls, but to a slight extent (3 animals) or not at all (2 animals) in the "operated" animals. In the second series of experiments,

[58] Stadie, W. C., and Van Slyke, D. D., *Arch. Intern. Med.*, 1920, xxv, 693.
[59] Mathews, S. A., and Nelson, C. F., *J. Biol. Chem.*, 1914, xix, 229.
[60] Rich, A. R., *Bull. Johns Hopkins Hosp.*, 1923, xxxiv, 321.
[61] Fiske, C. H., and Sumner, J. B., *J. Biol. Chem.*, 1914, xviii, 285.

the duration was only one hour, and comparable though not so marked increases in urea content were obtained in blood and muscle for both control and "operated" animals. The subjects of these shorter experiments were not noticeably abnormal at the time of the second taking of blood and tissue samples. As evidence that blood circulation in the liver was effectively blocked, an attempt on one "operated" cat to infuse the liver with gelatin colored with lead chromate by injection into the aorta and the superior mesenteric vein gave entirely negative results, while with two other "operated" cats analyses of the liver before and after the amino acid injection failed to reveal any increase in the total non-protein nitrogen, although a considerable increase was demonstrable in the control cats.

The experimental results of Fiske and Sumner indicate not only that other tissues than the liver are capable of forming urea, but that the liver must occupy a distinctly subordinate position in this respect, since isolation of this organ produces no effect on the rate of urea accumulation in the blood. It is somewhat unfortunate that ammonia determinations were not run on all samples. The investigators state that a few ammonia determinations were made, sufficient to convince themselves that variations in the urea results could not be attributed to ammonia, but these determinations were unsatisfactory and were not reported. The results for urea nitrogen therefore include ammonia nitrogen. It is further to be noted that the increases in the urea nitrogen in the blood in the injection experiments were always, and generally considerably, greater than the increases in the same constituent in the muscles, a relation somewhat difficult to reconcile with the conclusion that the latter stand in a causal relation to the former.

It seems evident that successful hepatectomy, in which all liver tissue is removed from the body and after which the animal remains to all appearances normal for several hours, is a more satisfactory and effective method of approaching all problems relating to liver function than an uncertain circulatory isolation of the liver. Recently at the Mayo Foundation a method of extirpating the liver has been worked out by Mann [62] which seems to satisfy the most exacting requirements. The success of the method is attributed to two factors: (*a*) The total removal of the liver, including that portion of the vena cava which it surrounds, is effected in a manner comparatively free from complications and such that the venous return from the other viscera and the lower extremities is not impaired, and (*b*) the discovery that the removal of the liver lowers the level of blood sugar below the point

[62] Mann, F. C., *Amer. J. Med. Sci.*, 1921, clxi, 37; *Amer. J. Physiol.*, 1921, lv, 285.

compatible with life and that hepatectomized animals could be kept alive and in normal condition, according to all outward appearances, for as long as twenty-four hours by the simple expedient of administering glucose in amounts sufficient to maintain a normal level of sugar in the blood. Recovery from the immediate effects of the operation is rapid. "Within an hour the animal is to all appearances normal; it walks around, responds to call, and exhibits the usual interest in its surroundings."

The results of extirpating the liver have been studied by Mann and his associates with several different species of animals, *i.e.*, dogs, geese,

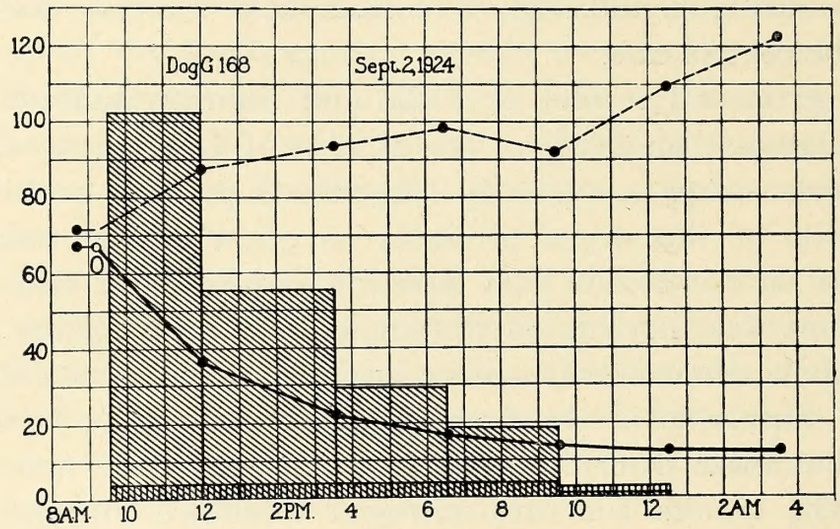


FIG. 19.—Blood and Urine Changes Following Complete Removal of the Liver.

The curve with the broken line shows the amino-acid nitrogen of the blood following complete removal of the liver, 0. The urea nitrogen in the blood is shown by the unbroken line. The amino-acid nitrogen content of the urine is shown by the rectangles with vertical hatching. The urea nitrogen plus ammonia nitrogen of the urine is shown by the rectangles with angle hatching. The height of the rectangles indicates the number of milligrams excreted each hour during the period indicated by the width of the rectangle. The blood urea nitrogen and amino-acid nitrogen are expressed in milligrams for each 1000 c.c. of blood. From the experiments of Bollman, Mann, and Magath (*Amer. J. Physiol.*, 1926, lxxviii, 259).

ducks, frogs, turtles, and fishes. The general effects are said to be the same in all species,[63] but a complete report has, at the present time, been confined to the results obtained with dogs. These results have been published by Bollman, Mann, and Magath.[57] They constitute an impressive array of evidence for the view that "the production of urea in the canine organism is entirely dependent upon the presence of the liver, since urea formation ceases completely as soon as the liver is removed."

Three series of experiments were performed. The first involved 75 dogs in which the liver only was removed. These animals lived from

[63] Mann, F. C., *J. Amer. Med. Assoc.*, 1925, lxxxv, 1472.

six to thirty-four and one-half hours after the operation. The second series involved a group of 12 dogs, in which the liver and kidneys were removed at the same time, and 3 dogs in which the entire contents of the peritoneal cavity were removed at the same time. These animals lived from five to twelve hours after the operation. In the third series of experiments both kidneys were removed from two dogs eight and twenty-four hours before the liver was removed. These animals lived for three and eight hours after hepatectomy. The experimental observations included chemical examination of blood, urine, and tissues according to approved methods of analysis.

The results of these admirable experiments have been summarized by Mann [63] as follows, in so far as they refer to amino acid metabolism:

"The urea content of the blood following removal of the liver depends entirely on the amount excreted from the body. If the kidneys are removed at the same time as the liver, or if the animal becomes anuric after hepatectomy, the urea content of the blood remains the same or falls slightly below its preoperative level. The decrease noted in such experiments can be accounted for by the amount lost in the saliva and vomitus. If the secretion of urine is maintained after hepatectomy, there is a marked decrease, not only in the urea content of the blood, but also in the amount eliminated through the urine and in the urea content of the tissues of the body, as the muscles. If the kidneys are removed from 8 to 24 hours before the liver is removed, so that at the time of hepatectomy the blood urea level is far above normal, no further increase in blood urea takes place. The urea found in the blood and tissues and excreted after operation accounts for all the urea present at the time of operation. These results conclusively prove that the production of urea absolutely ceases after removal of the liver. . . ."

"Removal of the liver is followed by a progressive increase in the amino-acid content of the blood. If the kidneys are removed at the same time as the liver, the amino-acids increase more rapidly in the blood. One amino-acid, glycocoll, has been injected intravenously to determine whether it would in any measure take the place of glucose, either in preventing the development of the hypoglycemic symptoms or in restoring the animal to normal after they had developed. Glycocoll never produced any beneficial action. It would thus seem that it could not in any measure take the place of glucose in the hepatectomized animal. There was never any evidence of the formation of urea following the injection of amino-acids into the hepatectomized animal. These observations, taken as a whole, would imply that the normal mechanism for the utilization of amino acids is probably destroyed by the removal of the liver. . . ."

"The result of experiments on the effect of removing the liver on the formation of ammonia are not entirely satisfactory. There is a shift in the relation of urea and ammonia nitrogen in the urine, with a relative increase in ammonia nitrogen compared to the decrease in urea nitrogen. There does not appear to be an increase in the total amount of ammonia in the urine or in the tissue. The liver probably does not have any special part in the production of ammonia. . . ."

The results by Bollman, Mann and Magath with reference to amino acid utilization in the absence of the liver are in agreement with some recent results reported by Gottschalk and Nonnenbruch [64] on men, involving urinary and blood analysis investigations after oral as compared with rectal administration of an amino acid mixture. The signifi-

[64] Gottschalk, A., and Nonnenbruch, W., *Arch. exper. Path. Pharm.*, 1923, xcix, 300.

cance of the experiments with reference to the liver function resides in the fact that the veins from the lower levels of the large intestine empty directly into the inferior vena cava instead of into the portal circulation leading to the liver. While amino acid utilization was normal with oral amino acid feeding as indicated by no appreciable increase in the amino acid nitrogen in the urine, in the case of rectal feeding

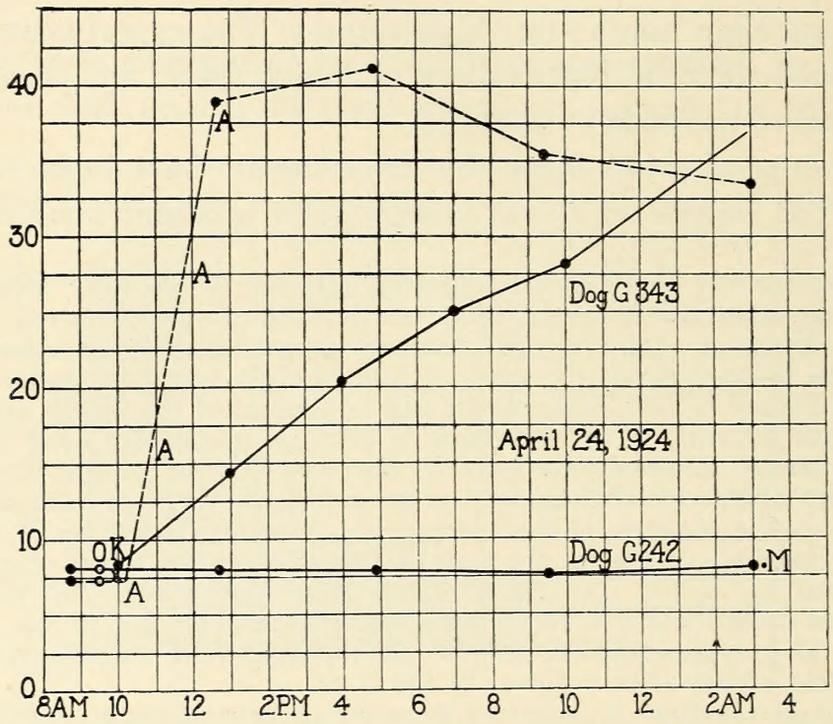


FIG. 20.—Effect of Total Removal of the Liver on Deamination.

The curves show the effect of intravenous injections, A, of 250 mgms. of glycine for each kilogram of body weight after total removal of the liver and of both kidneys, OK, in dog G242. The amino-acid nitrogen content of the blood (broken line) and the urea nitrogen of the blood (unbroken line) are expressed in milligrams per cent. M indicates the urea nitrogen content of the muscles in milligrams for each 100 grams of muscle. Comparison of these results is made with the effect of bilateral nephrectomy alone, K, on the blood urea nitrogen of dog G343 without the injection of amino acids. From the experiments of Bollman, Mann, and Magath (*Am. J. Physiol.*, 1926, lxxviii, 262).

there was a large increase in the amino acid nitrogen of the urine, so that this fraction of the total urinary nitrogen rose from a normal level of 4 to 6 per cent to about 26 per cent.

It is a difficult matter to weigh the evidence relating to the importance of the liver in urea formation and to reconcile all of it with any definite conclusion. Much of it can be ruled out, because of the recognized inadequacy of the chemical or other methods employed. If attention is confined to the work of the last ten or fifteen years, there can be no reasonable doubt but that the great preponderance of evidence speaks for the conclusion that the liver is the most important source, if

not practically the sole source of urea, in the mammalian organism at least. The work of Bollman, Mann, and Magath constitutes in itself a conclusive proof that, in the dog at least, urea formation ceases immediately upon removal of the liver. The prompt inhibition of urea formation observed is good evidence for the belief that the effect is direct rather than indirect through impairment of some other organ. Furthermore, deamination of amino acids does not seem to occur in the absence of the liver. It is, however, well to remember that the experiments of Bollman, Mann and Magath are in good agreement with the preponderance of experimental evidence preceding them. They

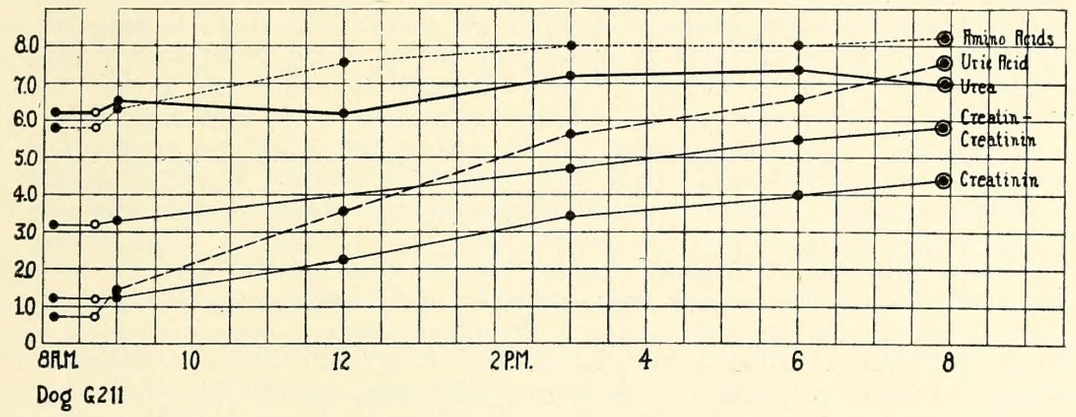


FIG. 21.—Uric Acid in the Blood Following Total Removal of the Liver.

Curve showing the changes in the blood of a hepatectomized dog with bilateral nephrectomy. 0, liver and kidneys were removed. Uric acid, creatine-creatinine, and creatinine are expressed in milligrams per each 100 c.c. Amino acids and urea are expressed in milligrams of nitrogen for each 100 c.c. From the experiments of Bollman, Mann, and Magath (*Amer. J. Physiol.*, 1925, lxxii, 633).

are not revolutionary in their significance as might well be supposed in some quarters. Whether the conclusions supported by the experiments on dogs apply to the lower animals is doubtful if one may judge from the reported experiments of Gottschalk and Nonnenbruch [65] on toads and frogs. In these investigations, injection of amino acids into the dorsal lymph sac caused distinct and closely equal increases in the urea nitrogen of the blood (obtained by heart puncture) in normal frogs and toads and in frogs and toads from which the liver had been removed completely or had been poisoned by phosphorus. On the other hand, Falkenhausen and Siwon,[66] in experiments on liver extirpation in geese and the blood changes following amino acid injections, obtained evidences of ammonia formation but no evidence of urea formation. The urea content of the blood decreased progressively after removal of the liver, in agreement with the findings of Bollman, Mann and Magath.

[65] Gottschalk, A., and Nonnenbruch, W., *Arch. exper. Path. Pharm.*, 1923, xcix, 261.
[66] Falkenhausen, M. F., and Siwon, P., *Arch. exper. Path. Pharm.*, 1925, cvi, 126.

Van Slyke [67] has formulated a very peculiar theory of the function of the liver with reference to urea formation, based largely upon his experimental findings that liver tissue, on the one hand, possesses a greater avidity for amino acids than muscle tissue, and, on the other hand, disposes of its temporary store of amino acids much more rapidly than muscle tissue. The following quotation will serve to explain Van Slyke's views, which have stimulated considerable discussion and experimental investigation:

> . . . The next question to be raised is, does the liver, during the digestion of a protein meal, wait till the other tissues are saturated with amino-acids and then begin to destroy the unnecessary excess, which is not needed by the organism, or does it begin to destroy the first that reach it in the portal blood? Unreasonable as it may seem, the latter behavior is what we observe. In order to test this point, urea determinations were made at short intervals on blood from dogs which, after two days' fast, had received heavy meals of meat. It was found in all cases that the urea began to rise almost immediately after the meat was consumed. There was no interval of waiting commensurate with what might be expected if urea formation were delayed until after the tissues in general had replenished their store of amino-acids. It was furthermore shown by roentgenograms that the blood urea began to rise at almost the minute the first particle of food passed from the stomach into the duodenum. Since London has shown that no absorption occurs until the chyme enters the intestine, our results indicate that the very advance guard of amino-acids entering the blood after a protein meal is, in part at least, immediately turned into urea. The interval between feeding and the beginning of urea formation is so short that this conclusion would be really forced on us, even without the Roentgen-ray evidence. Unreasonable as it appears, the organism does not wait until it has absorbed sufficient protein digestion products to meet its immediate requirements, and thereafter begin to turn the surplus into urea. The very beginning of absorption stimulates the urea-forming function into activity. This behavior explains the fact that no matter how depleted by disease or hunger the tissues of an individual may be, the greater part of the protein nitrogen which he may subsequently consume is excreted as urea, only a small portion being retained to rebuild the wasted tissues.
>
> That urea is the form taken by all of the amino-acid nitrogen which disappears in the liver does not absolutely follow from our results. Pflüger was of the opinion that the liver cells store reserve protein from the food, just as they store reserve carbohydrate in the form of glycogen. Our results do not at present exclude this possibility. In order to do so, we should have to prove that the liver gives out as urea an amount of nitrogen exactly equal to that which it absorbs as amino-acid, and our experimental technic does not yet enable us to say whether or not this is the case. The analytic methods are adequate, but the fact that the amino-acids are held for a certain time before they are destroyed and that the urea also may not pass instantly from the liver tissues to the hepatic vein makes the striking of an exact balance between amino-acid intake and urea outgo a matter of experimental difficulty which has not yet been overcome. The possibility, therefore, remains open, tho certainly not proved, that some of the amino-acids may be converted by the liver into a form of reserve protein which is stored like glycogen.

The above interpretation of the experimental results obtained seems open to question. In the first place the formation of urea by the liver from ammonium salts arising from the hydrolysis of protein in the stomach and intestine is not considered. The increase observed in the

[67] Van Slyke, D. D., *Arch. Intern. Med.*, 1917, xix, 56. *See also* The Harvey Lectures, 1915-16, xi, 146.

concentration of urea in the blood cannot with certainty, therefore, be ascribed to the deamination of amino acids, and such an interpretation may even be considered improbable in view of the close coincidence observed between the appearance of surplus urea in the blood and the first passage of chyme from the stomach to the duodenum. In all probability amino acids are not liberated during the gastric digestion of proteins in any but negligible quantities, while ammonia is undoubtedly one of the products of peptic digestion and is produced in considerable quantities from most proteins. Hence any urea formed by the liver immediately after the entrance of chyme into the duodenum more probably originated from ammonium salts than from amino acids.

The rate of increase of urea in the blood during protein digestion can give no sure clew to the rate of amino-acid catabolism, nor to the time at which this catabolism is initiated. The liver is probably competent to keep the concentration of ammonia in the systemic blood and in the other tissues within very narrow limits under normal conditions of metabolism, by conversion of ammonium salts into urea; that it is competent to do likewise with the concentration of amino acids is contradicted by the amino-acidemia readily produced during protein digestion. In fact, if the liver possessed the capacity of deaminizing any large share of the incoming amino acids, many phases of protein utilization, especially in the growing animal, would be difficult to understand.

It should be noted that the conclusion of the predominance of the liver in urea formation, based upon the work of Bollman, Mann and Magath and others, does not necessarily involve an indorsement of the view of Van Slyke that deamination starts in the liver as soon as amino acids reach it in the portal blood. A delayed formation of urea in the liver until the other tissues of the body have had the opportunity for a maximum utilization of amino acids in anabolism is more compatible with the known facts of amino-acid metabolism and utilization than is the immediate formation postulated by Van Slyke. Folin and Berglund [68] have stated that if Van Slyke's theory is correct, then during the early stages of absorption of amino acids the increase in the urea content of the blood must *precede* the increase in the content of amino acids. Their own experimental results on moderate protein feeding in human subjects do not indicate such a relation, although certain results on dogs reported later by Morgulis [69] do conform to this expectation. However, the reasoning of Folin and Berglund revealed in this statement seems somewhat inconsistent with the objection they have them-

[68] Folin, O., and Berglund, H., *J. Biol. Chem.*, 1922, li, 395.
[69] Morgulis, S., *J. Biol. Chem.*, 1925, lxvi, 353.

selves raised to Van Slyke's experimental findings analogous to those of Morgulis. In any case the time relations of changes in the urea and amino acid contents of the systemic blood during protein digestion and absorption do not necessarily have any direct bearing upon the general question of the predominance of the liver in deamination and urea formation, though they may be directly related to some particular theory of liver function.

The question of urea formation has been related mainly to the α-amino grouping of amino acids. However, the nitrogen contained in other groupings, such as the pyrrole group in proline, the guanidine group in arginine, the imidazole group in histidine, and the indole group in tryptophane, may also be entirely converted into urea in catabolism and excreted in the urine. The processes involved must evidently be complicated and not susceptible of a general description. In so far as available information permits, they will be considered in connection with the special phases of amino acid catabolism.

THE AMMONIA CONTENT OF THE BLOOD AND THE EXCRETION OF AMMONIA IN THE URINE

Although the evidence seems clear that no other tissue than liver tissue is normally concerned to an appreciable extent in urea formation, nothing can be said with any degree of confidence concerning the locus of deamination, if this function is at all localized. Perfusion experiments on the liver, such as those concerned with the production of α-ketonic and α-hydroxy acids and of acetoacetic acid from amino acids, have shown conclusively that this organ is capable of liberating the amino group from α-amino acids, but definite evidence concerned with the capacity of other tissues of promoting the same reaction is not at hand. It may be recalled in this connection that the amino acid content of the blood, normally so constant in the post-absorptive period, even in most pathological conditions,[70] rises progressively after complete extirpation of the liver, according to Bollman, Mann and Magath, while no increases in the ammonia excretion or in the ammonia content of the tissues were observed. These findings indicate, though hardly prove, that deamination is largely if not entirely confined to the liver. If all tissues were concerned in deamination and only the liver in urea formation, one might expect to find considerable amounts of ammonia in the blood at all times, though particularly during digestion, bound to the kidney for excretion or to the liver for conversion into harmless urea. On the other hand, if the liver only, or mainly, were concerned

[70] Greene, C. H., Sandiford, K., and Ross, H. *J. Biol. Chem.*, 1924, lviii, 845. *See also* Feinblatt, H. M., and Shapiro, I., *Arch. Intern. Med.*, 1924, xxxiv, 690.

in deamination, the ammonia content of the blood at all times would be minimal, representing possibly only an inevitable leakage of ammonia from the liver. In view of the marked toxicity of ammonium salts [71] after gaining access to the systemic blood, it might be considered a wise provision that tissues concerned in ammonia formation should also possess the capacity of detoxicating the ammonia formed by conversion into urea.

A review of the literature up to 1921 upon the concentration of ammonia in blood has recently been published by Nash and Benedict [72] and need not be considered here. It discloses much confusion in the values reported and a general trend downward with the increasing appreciation of the difficulties involved in the determination, due mainly to labile ammonia-yielding constituents in the blood and to contamination of the chemical reagents used with ammonium salts. The first analysts to overcome the difficulties encountered were Folin and Denis,[73] who in 1912 published a notable contribution on the subject, demonstrating an ammonia concentration of the arterial blood of cats no greater than 0.03 to 0.08 mgm. of ammonia nitrogen per 100 cc. While these low values have not gone unchallenged by other biochemists, even when professedly using the analytical method of Folin and Denis, they were notably confirmed in 1917 by Barnett [74] who used a micro-aëration-titration method of his own. Barnett demonstrated, in agreement with experiments of Medwedew [75] published six years earlier, that the ammonia content of drawn blood increased rapidly on standing, so that after 30 minutes standing the concentration is two to three times as great as that of blood aërated immediately. The need of immediate aëration as soon as possible after the blood is drawn, and of completing the analysis as rapidly as possible, is clearly indicated if values even approaching those of the preformed ammonia in blood are to be obtained. This experimental finding has been confirmed by Parnas.[76]

If the blood ammonia is as low in concentration as these minimal figures indicate, it is difficult, as Nash and Benedict prove, to account for the normal excretion of ammonia in the urine, to say nothing of the greatly increased excretion found in certain pathological conditions or after acid ingestion. Either these minimal values are actually too low, due, for example, to incomplete aëration, or the kidney itself is able to produce the ammonia it excretes, possibly from the labile ammonia-

[71] Underhill, F. P., and Kapsinow, R., *J. Biol. Chem.*, 1922, liv, 451.
[72] Nash, T. P., Jr., and Benedict, S. R., *J. Biol. Chem.*, 1921, xlviii, 463.
[73] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 161, 527.
[74] Barnett, G. D., *J. Biol. Chem.*, 1917, xxix, 459.
[75] Medwedew, A., *Z. physiol. Chem.*, 1911, lxxii, 410.
[76] Parnas, J. K., *Biochem. Z.*, 1924, clii, 1; *ibid.*, 1925, clv, 247.

yielding blood constituents responsible for the increasing concentration of ammonia in drawn blood on standing. As the result of a series of carefully performed experiments, mainly on dogs, Nash and Benedict confirmed the low blood ammonia values reported by Folin and Denis and by Barnett, and formulated the astounding conclusion, not only that the kidney itself forms the ammonia it excretes, but that the kidney is the center of ammonia production in the body. The ammonia in the blood, therefore, is simply due to a leakage from the kidney, and is kept at a minimal concentration possibly by the liver, which would withdraw excess ammonia and convert it into urea.

While the work of Folin and Denis furnished the analytical tool requisite for investigating the physiological significance of the ammonia content of the blood, their work tended to discourage such investigation, because the mere traces of ammonia shown to be present in systemic blood were taken to mean that blood ammonia is "a rather unimportant feature of normal metabolism." On the other hand, the outcome of the investigations of Nash and Benedict, because of their unexpectedness, have attracted widespread attention and have apparently furnished the impetus for considerable subsequent research. The experimental findings of Nash and Benedict, from which their conclusions were deduced, call for further consideration. They may be summarized briefly as follows:

1. Drawn blood, after a slight increase in ammonia content during the first 30 minutes' standing, shows no further change in this constituent. Plasma showed no such increase. The increase noted with whole blood was therefore explained as due to hemolysis and perhaps to diffusion of ammonium salts from the corpuscles into the plasma. No confirmation was obtained of the results of previous investigations concerning the presence of "labile ammonia-yielding bodies" in the blood.
2. The concentration of ammonia in blood is as low as the reported analyses of Folin and Denis and of Barnett indicate, *i.e.*, of the order of 0.1 mgm. of ammonia nitrogen per 100 cc.
3. The blood ammonia is not increased in concentration in conditions (phlorhizin diabetes in dogs) in which the excretion of urinary ammonia is higher than normal.
4. In the total absence of kidney function in dogs following double nephrectomy or ligation of both ureters, there is no accumulation of ammonia in the blood.
5. The concentration of ammonia in the blood of the renal vein is invariably greater (averaging twice as great) than that of the carotid artery or of the vena cava, while the concentration of ammonia in the blood of the vena cava was substantially the same as that of the carotid artery.
6. The injection of acid intravenously or of alkali subcutaneously has no influence on the ammonia content of the systemic blood.

At their face value these results obviously justify the conclusion that urinary ammonia is produced in the kidney, and at least indicate that blood ammonia is the result simply of an over-production of ammonia in this organ. Since they were unable to obtain evidence of the presence in

blood of "labile ammonia-yielding bodies," Nash and Benedict incline to the view that urea is the source of both urinary and blood ammonia, although Wakeman and Dakin [77] were unable to obtain evidence of the reversibility of the urea-producing reaction in the liver. However, Barnett and Addis [78] have noted marked increases in blood ammonia in rabbits following intravenous injection of large doses of urea, indicating the possibility of ammonia production from urea.

There has been considerable confirmation of the results and conclusions of Nash and Benedict. The decrease observed in the excretion of urinary ammonia in uranium nephritis is cited by Hendrix and Bodansky [79] as support for the theory of Nash and Benedict, while the same significance is attached by Russell [80] to her demonstration that there is no retention of ammonia in the blood in severe clinical nephritis. Loeb, Atchley and Benedict [81] found that in the dog, normally secreting considerable amounts of ammonia in the urine, the ammonia content of the blood in the renal vein (0.17 mgm. NH_3-N per 100 cc.) was about four times that of the blood in the femoral artery (0.04 mgm. NH_3-N per 100 cc.), while in the rabbit, normally secreting but traces of ammonia in the urine, even after acid injection, the blood of the renal vein and that of the aorta contained practically the same amount of ammonia (0.069 mgm. and 0.052 mgm. of NH_3-N per 100 cc. respectively). In this investigation, however, the blood of the vena cava in dogs was invariably (3 cases) found to contain more ammonia than that of the femoral artery, while in one dog splenic venous blood also was higher in ammonia content than arterial blood. These results suggest that some ammonia is added to the blood by other tissues than the kidney. Henriques [82] found no significant differences among the ammonia contents of the blood of the carotid, vena cava, and renal vein in dogs, even after the injection of 50 to 70 cc. of 0.1 N-HCl to stimulate ammonia production. However, with the same method Henriques and Christiansen [83] were earlier unable to demonstrate a greater ammonia content in the portal than in the systemic blood, while in a later investigation, using a different method, Henriques and Gottlieb [84] conclude that there is very probably no preformed ammonia in the blood. Hence, obviously the urinary ammonia must be manufactured in the kidney. It is, perhaps,

[77] Wakeman, A. J., and Dakin, H. D., *J. Biol. Chem.*, 1911, ix, 327.
[78] Barnett, G. D., and Addis, T., *J. Biol. Chem.*, 1917, xxx, 41. *See also* Adolph, E. F., *Proc. Amer. Physiol. Soc., Amer. J. Physiol.*, 1925, lxxii, 185.
[79] Hendrix, B. M., and Bodansky, M., *J. Biol. Chem.*, 1924, lx, 657.
[80] Russell, D. S., *Biochem. J.*, 1923, xvii, 72.
[81] Loeb, R. F., Atchley, D. W., and Benedict, E. M., *J. Biol. Chem.*, 1924, lx, 491.
[82] Henriques, V., *Z. physiol. Chem.*, 1923, cxxx, 39.
[83] Henriques, V., and Christiansen, E., *Biochem. Z.*, 1917. lxxx, 297.
[84] Henriques, V., and Gottlieb, E., *Z. physiol. Chem.*, 1924, cxxxviii, 254.

a fair conclusion that the work of Henriques and associates has added little to the question of the origin of blood ammonia.

A most significant contribution on ammonia formation has recently been reported by Bliss [85] from Folin's laboratory. In many respects the experimental data obtained by Bliss are in good agreement with those obtained by Nash and Benedict, but nevertheless he is compelled to disagree with their conclusions. Bliss confirmed the finding of Nash and Benedict that renal venous blood in dogs contains twice as much ammonia as arterial (carotid) blood (0.23 mgm. NH_3-N as compared with 0.11 mgm.). Also, he found that venous blood coming from a purely muscular region (femoral vein) contains no more ammonia than the arterial blood (femoral artery). Also, no difference in ammonia content was found between carotid and jugular blood. However, a comparison of the ammonia content of the blood of the pancreatico-duodenal vein with that of carotid blood showed the same relation as was observed for the kidney, *i.e.,* twice as much ammonia in the blood coming from the pancreas and the (clean) duodenum as in arterial blood. Splenic venous blood also was found to be relatively high in ammonia, so that the evidence of Bliss confirms that of Loeb, Atchley and Benedict in indicating that other glands than the kidney are normally contributing ammonia to the blood.

If other organs than the kidneys produce ammonia and excrete it into the blood, it would be expected, in contradiction to the results of Nash and Benedict, that double nephrectomy would lead to a retention of ammonia, as well as other metabolites, in the blood, *unless the nephrectomized animal possessed some means of preventing an accumulation of ammonia in the blood.* Bliss found that in vomiting the nephrectomized animal possesses an effective mechanism for removing ammonia from its blood. Observation of nephrectomized or nephritic dogs proves that alternate drinking and vomiting is a natural phenomenon, and analysis of the vomitus showed it to contain considerable quantities of ammonia. In nephrectomized animals, the course pursued by the ammonia content of the blood depended entirely upon this vomiting reflex. If vomiting occurred with sufficient frequency, enough ammonia was removed from the body by this path so that the ammonia content of the blood remained unchanged. With less frequent vomiting an irregular increase and decrease in blood ammonia values, the latter coincident with vomiting, was observed, while the absence of vomiting led to a rapid increase in blood ammonia. It was also found that injection of ammonium carbonate or of urease into the blood of dogs could induce vomiting. Bliss suggests

[85] Bliss, S., *J. Biol. Chem.,* 1926, lxvii, 109.

that in the kidney-functionless dogs of Nash and Benedict, vomiting might have occurred with sufficient frequency to insure against significant increases in blood ammonia. Finally, Bliss found that in human nephritics, vomiting plays as significant a rôle in the maintenance of normal blood ammonia values as in nephrectomized dogs.*

Thus the problems opened up by the investigations of Nash and Benedict appear to have been settled in a manner contrary to their own conclusions. Indeed, the conclusion that kidney tissue only is capable of forming ammonia is quite anomalous in view of contemporary and preceding work. We may cite further in this connection the investigations of Tashiro and his associates [86] indicating that fresh surviving nerves and muscles continuously excrete a volatile base which is presumably ammonia. The conclusion seems justified, therefore, that most if not

* In a later article, Benedict and Nash (*J. Biol. Chem.*, 1926, lxix, 381) offer a reply to the paper of Bliss. Their most significant statement is that their original investigation was concerned solely with the formation of urinary ammonia, which is produced for the neutralization of non-volatile acid. It is true that their problem as stated in the introduction to their first paper was thus restricted. It is also true that their results establishing a concentration of ammonia in blood coming from the kidney twice as great as that in the blood going to the kidney, finally and adequately demonstrated that the urinary ammonia is formed in the kidney. If the net effect of the circulation on the kidney, as far as ammonia is concerned, is to subtract rather than to add, then obviously the kidney is producing more ammonia than that appearing in the urine. This finding was confirmed by Loeb, Atchley and Benedict, as well as by Bliss. Even when the excretion of urinary ammonia is practically suppressed by alkali treatment, the kidney continues to add ammonia in relatively considerable amounts to the blood.

However, in their other experiments, Nash and Benedict appeared to be investigating the general problem of ammonia formation in the tissues, and explicitly state (p. 483) that "the kidney is the center of ammonia production in the body," and that "we must assume that the ammonia of the systemic blood represents an equilibrium state between the ammonia which comes into the circulation by way of the renal veins (and possibly traces of ammonia from the intestinal circulation which pass the liver) and the transformation of this ammonia into urea." On this general question, their results may reasonably be interpreted as merely a demonstration of the efficiency of the liver in maintaining a low (and safe) level of ammonia in the blood. Löffler [37] has shown that even in the presence of inorganic acids the surviving liver will convert ammonium salts of such acids into urea.

Their objections to much of Bliss' data are largely unconvincing. Against their view that the pancreatic blood analyzed by Bliss was seriously contaminated with duodenal blood, may be cited the fact that the difference observed in the ammonia content of pancreatic and systemic blood was quite unrelated to the digestive condition of the dog, whether fed or fasting. It is true that the difference observed by Bliss between the ammonia in splenic and in systemic blood was slight, still it was fairly general (nine cases out of fourteen, three of which showed no difference). The similarity between the ammonia content of femoral or jugular venous blood and that of carotid blood may mean a relatively slow production of ammonia by the muscles that is adequately taken care of by the withdrawal of ammonia by the liver and its conversion into urea. Benedict and Nash point out that the amounts of ammonia recovered by Bliss from the vomitus of nephritic and uremic dogs are at times much greater than could have originated from the blood. They suggest contamination with ammonia-containing material from the large intestine, but any appreciable contamination of this character seems highly improbable. Since Bliss was evidently aware of the possibility of contamination from the duodenum and investigated it with entirely negative findings, the probability of any gross error of this nature appears small. While all of the ammonia of the vomitus of nephritic or kidney-functionless dogs may not have been contributed by the blood, it nevertheless appears to be true that some must have been so contributed. In this connection Benedict and Nash fail to consider the synchronous relation between reductions in blood ammonia and vomiting, indicating a causal connection, and also the fact that with hospital patients, the vomitus from nephritic subjects contained considerable amounts of ammonia while that from non-nephritic subjects was practically ammonia-free. It appears, therefore, that Bliss' interesting theory of the function of vomiting in nephritis has not been greatly weakened by the discussion of Benedict and Nash.

A recent note by Bliss (*Proc. Amer. Soc. Biol. Chem.*, 1928, ix) contains the claim that there is a combined form of ammonia in blood equivalent to 118 to 125 mgms. of nitrogen per 100 cc., and that the kidney contains an enzyme capable of splitting free ammonia from this compound. The value to be placed upon this revolutionary finding must await a full report of the work.

[86] Tashiro, S., *Amer. J. Physiol.*, 1922, lx, 519; Lee, O. P., and Tashiro, S., *Amer. J. Physiol.*, 1922, lxi, 244.

all of the tissues in the body produce ammonia, but that its detoxication by conversion into urea appears to be sharply localized in the liver.

THE RELATION OF AMMONIA FORMATION TO THE REGULATION OF THE NEUTRALITY OF THE TISSUES

The normal performance of the metabolic processes in animal tissues is dependent to a high degree on the maintenance of a chemical reaction close to neutrality. The blood in the body is normally slightly alkaline (about pH = 7.4), while in the normal individual, according to Wilson,[87] the extreme variations in the hydrogen ion concentration of the blood thus far reported in the literature are represented by a pH of 7.05 after short periods of strenuous muscular exercise, and, on the alkaline side, by a pH of 7.85 obtained after forced breathing. Although the limits of reaction compatible with life are still in doubt, blood as acid as pH 6.95 and blood as alkaline as pH 9.00 has been observed. The reaction of the cell fluids is difficult to determine, but it appears probable that it does not differ greatly from that of blood; if one may judge from a comparison of the pH of blood plasma and blood corpuscles, the cell fluids are slightly more acid than the blood by about pH 0.05 to 0.13. However, as the blood becomes more acid this difference in reaction decreases until at pH 6.5 it is said to disappear entirely.

This relative constancy in the reaction of the body fluids is maintained in spite of a continuous production of acids in metabolism and a continuous inflow of nutriment from the alimentary canal which, upon oxidation in the tissues yields an inorganic residue of widely varying acidity or basicity. The main acid metabolite, carbon dioxide, is volatile and is readily disposed of in the lungs provided the reaction of the blood is sufficiently on the alkaline side to permit of its ready transportation. The constant availability of carbon dioxide in the tissues also permits of a ready means of disposal as carbonates by way of the kidneys of any excess base arising from the food. However, the continuous production of non-volatile acids in the metabolism of protein (mainly phosphoric and sulfuric acids) constitutes a greater strain upon the neutrality regulating mechanism.

The first defense against a serious disturbance of the hydrogen-ion concentration of the tissues by the continuous production of acids is the presence in the blood and body fluids of a very effective buffer mixture consisting of proteins and acid and basic salts of carbonic and phosphoric acids. This is obviously only a temporary expedient, since the alkali reserve of the tissues would be rapidly depleted if no mechanism were

[87] Wilson, D. W., *Physiol. Rev.*, 1923, iii, 295.

at hand to return the system to its normal condition. This final mechanism resides in the kidneys. This function of the kidney has been studied in great detail by Henderson and Palmer, and their work as reviewed by Wilson constitutes the basis of the following brief discussion.

"Normal human urine ranges in reaction from pH 4.8 to 7.4. At these reactions the strong acids, such as sulfuric and phosphoric, cannot be eliminated as such but only in the form of salts. At pH 4.8, 1 mol of sulfuric acid is combined with nearly two mols of base. One mol of phosphoric acid is combined with a little less than one mol of base (NaH_2PO_4), whereas in the body where the reaction is more alkaline, the phosphoric acid is combined with 1.8 mols of base. It is evident, then, that in the excretion of 1 mol of phosphoric acid, more than 0.8 mol. of base may be retained by the body and serve to neutralize other molecules of acid. If, however, there is an excess of base in the body, it may be excreted as disodium phosphate or bicarbonate and a decrease in the C_H of the urine will result." Although the reaction of the urine is an index of the output of acid, it does not furnish much evidence concerning the quantity of acid excreted. Such information can only be determined by titration of the urine using suitable indicators.

The excretion of an acid urine constitutes only one of the means available in the kidney for the protection of the body against acid produced in metabolism or administered to it as such. Another means is the substitution of ammonia produced in the deamination of amino acids, for some of the fixed base, permitting the elimination of acids in the form of neutral salts without the loss of fixed base from the body. Each mol of ammonia thus used constitutes a saving of 1 mol of fixed base. That this mechanism is entirely confined to the kidney seems probable at the present writing. It is a mechanism that is brought into play most readily in man and the carnivorous animals, and only to a lesser extent in the herbivorous animals.[88] The rabbit, in fact, practically possesses no capacity for using ammonia in this way. As a defense against acid administration it is very effective, particularly when the administration is by way of the stomach, according to the experiments of Keeton on dogs.[89] Fiske and Sokhey [90] working with cats have confirmed Keeton's results in showing that acid administered *per os* may be excreted entirely as the ammonium salt, though generally the ammonia accounts for only 50 to 70 per cent of the acid. Acid given subcutaneously was about as effectively handled by ammonia neutralization as when given *per os,* though

[88] Steenbock, H., Nelson, V. E., and Hart, E. B., *Wisc. Agr. Exper. Sta., Res. Bull.* 36, 1915.
[89] Keeton, R. W., *J. Biol. Chem.,* 1921, xlix, 411.
[90] Fiske, C. H., and Sokhey, S. S., *J. Biol. Chem.,* 1925, lxiii, 309.

administration intravenously frequently led to a fall in urine ammonia or to the excretion of an alkaline urine. However, as Fiske and Sokhey point out, this difference in effect is in all probability related to the hyperpnea produced, causing an over-ventilation of the blood, and a liberation of fixed base equal in one experiment to 2.5 times the equivalent of the sulfuric acid injected. However, in the human organism the ammonia neutralization mechanism is not effective in all types of pathological acidosis, for example, in certain forms of nephritis, and in general the acid-forming mechanism of the kidney seems to be the more sensitive and elastic, as greater changes occur normally in the acid titration than in the ammonia excretion.

Although acid ingestion causes a prompt increase in ammonia excretion, the ingestion of an acid salt (NaH_2PO_4) does not,[91] while the ingestion of $NaHCO_3$ causes the ammonia excretion to diminish [92] and in large doses to disappear entirely.[93]

Excessive quantities of base can be removed easily by the kidney. The limit to this function seems to be merely that $NaHCO_3$ cannot be excreted in a solution more concentrated than 0.3 *N*. With sufficient quantities of water available, therefore, large quantities of base may be eliminated.

Although the relation of kidney function to blood composition is generally considered as one such that the former regulates the latter, it appears equally plausible that the composition of the blood regulates kidney function, and in the present case, the acid-base balance of the blood, as measured by its hydrogen-ion concentration, may be considered as regulating urine excretion. As the C_H of the blood increases, the C_H of the urine, its acid titration, and its ammonia content increases, and vice versa. According to Wilson [87] the differences in reaction between urine and blood plasma during an acid tide increases to a maximum when the urine has a pH of 4.7 and the blood a pH not much less than 7.0, while during an alkaline tide the difference decreases until the two fluids have the same reaction in the neighborhood of pH 7.8.

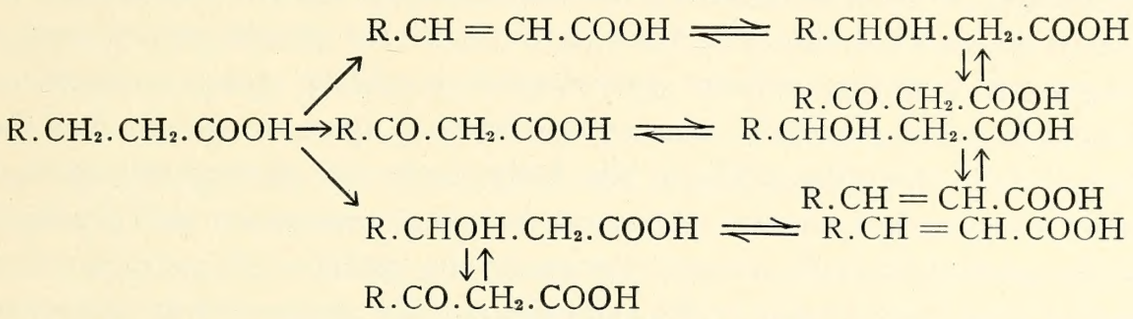
THE METABOLISM OF THE ACIDS RESULTING FROM DEAMINATION

The α-hydroxy or α-ketonic acids resulting from the deamination of amino acids may evidently be oxidized directly, or they may be converted into sugar and stored as glycogen, or converted into fats. In the former case, the first step would seem to be the oxidation of the α-carbon to a carboxyl group with the liberation of carbon dioxide and the formation

[91] Marriott, W. McK., and Howland, J., *Arch. Intern. Med.*, 1918, xxii, 477.
[92] Janney, N., *Z. physiol. Chem.*, 1911-12, lxxvi, 99.
[93] Davies, H. W., Haldane, J., and Kennaway, E. L., *J. Physiol.*, 1920, liv, 32.

of a fatty acid with one less carbon atom than the original amino acid. The fatty acid thus formed is then readily oxidized to carbon dioxide and water.

In case of straight-chain aliphatic acids, their catabolism apparently proceeds according to Knoop's scheme of β-oxidation, the β-carbon being successively oxidized with the successive liberation of two-carbon groups. According to Dakin,[3, 94] β-oxidation may occur with the direct formation of β-hydroxy, β-ketonic, or α-β unsaturated acids, or, more probably, all of these acids are in readily shifting equilibrium with each other and are easily interconvertible, according to the following scheme:



In the case of branched chain fatty acids, produced from the deamination of valine, leucine, and isoleucine, the side chain, apparently, is first removed, the residue then undergoing oxidation in the manner just indicated. In the case of the dibasic acids resulting from the deamination and α-oxidation of aspartic and glutamic acids, *i.e.,* malonic and succinic acids respectively, the method of oxidation is not clear. The special cases of cystine and arginine and of those amino acids containing cyclic structures will be considered in the following chapter.

THE CONVERSION OF AMINO ACIDS INTO SUGAR

The conversion of certain amino acids into glucose has long been known to occur in the diabetic organism. Ringer and Lusk [95] in particular have obtained very clear cut results by the use of fasting dogs made completely diabetic by phlorhizin injection. Their results indicate a complete conversion of the carbon of glycine and alanine into glucose, and a conversion of three of the carbons of aspartic and glutamic acid. On the basis of these and other results including his own, Dakin [3] makes the following summary of the possibilities of the conversion of amino acids into glucose

(1) "The amino acids from proteins which yield glucose freely in the diabetic organism are all those containing two, three, four and five carbon atoms, except valine.

(2) "Arginine is the only amino acid with more than five carbon atoms which

[94] Dakin, H. D., *J. Biol. Chem.,* 1923, lvi, 43.
[95] Ringer, A., and Lusk, G., *Z. physiol. Chem.,* 1910, lxvi, 106.

may furnish glucose freely, and in this case it evidently comes from ornithine with five carbon atoms into which it is converted by arginase.

(3) "All the straight chain amino acids yield sugar except lysine.

(4) "The amino acids with branched chains, including valine, leucine, and isoleucine, furnish little or no sugar.

(5) "Proline is the only cyclic amino acid known to yield much glucose. Hydroxyproline has not yet been investigated. The aromatic amino acids do not yield glucose.

(6) "The close structural relations between ornithine, proline, glutamic and β-hydroxyglutamic acids, all of which yield about three-fifths of their carbon as glucose, suggests that their catabolic paths may be similar, and it is not improbable that lactic acid may be an intermediate product."

The possibility of sugar formation from amino acids is of great significance to dietetic control in clinical diabetes. However, it should be clearly appreciated that the demonstration of the possibility of such a conversion under a definite pathological condition (experimental or clinical diabetes) has only an uncertain bearing upon the question of the normal fate of amino acids in the body. Even the demonstration that under more normal conditions protein *may* be transformed into glycogen in the animal body,[96] cannot, obviously, be interpreted to mean that glucose is an obligate step in the catabolism of the glycogenic amino acids.

The importance of the liver in the formation of sugar from amino acids has been shown in a very convincing and dramatic manner by Mann and Magath in their studies on the physiology of the liver by the method of hepatectomy. After the removal of the liver, the blood sugar level progressively decreases,[97] accompanied by a progressive decrease in muscular strength, until at a minimal concentration of 0.025 to 0.035 per cent, the animal dies in convulsion. The injection of sugar at this time or earlier defers this syndrome, apparently by raising the concentration of sugar in the blood.[98] The injection of glycogenic amino acids has no such effect.[63] Furthermore, the presence of the liver was shown to be essential to the hyperglycemia following pancreatectomy.[99] Following extirpation of the liver, the muscle glycogen also decreases, though the symptoms which appear at the critical hypoglycemic level bear no relation to the absolute amount of glycogen in the muscles, and may appear while there is still sufficient glycogen to bring the blood sugar to normal if it were released.[100] From these experiments the conclusion appears warranted that "the glycogen in the muscles is incapable of rapid conversion to glucose to maintain the level of sugar in the blood. The liver must be regarded as the source of glucose in the blood, and the muscles play little or no part in maintaining the normal level of blood sugar."

[96] Cramer, M., *Ergebn. Physiol.*, 1902, i, 803. *See also* Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1924, lix, 29.
[97] Mann, F. C., and Magath, T. B., *Arch. Intern. Med.*, 1922, xxx, 73.
[98] Mann, F. C., and Magath, T. B., *ibid.*, 1922, xxx, 171.
[99] Mann, F. C., and Magath, T. B., *ibid.*, 1923, xxxi, 797.
[100] Bollman, J. L., Mann, F. C., and Magath, T. B., *Amer. J. Physiol.*, 1925, lxxiv, 238.

KETOGENESIS AND ANTIKETOGENESIS

It is a fact of great interest, though of uncertain significance in normal metabolism, that those amino acids capable of yielding sugar in the diabetic organism do not yield acetoacetic acid, either in diabetes or on perfusion through a surviving liver, while several of the non-glucogenetic amino acids, *i.e.,* leucine, tyrosine, phenylalanine, and probably histidine, are ready acetoacetic-acid formers. For other amino acids, neither the possibility of glucose formation nor of acetoacetic acid formation has been demonstrated, *i.e.,* valine, isoleucine, lysine, and tryptophane.

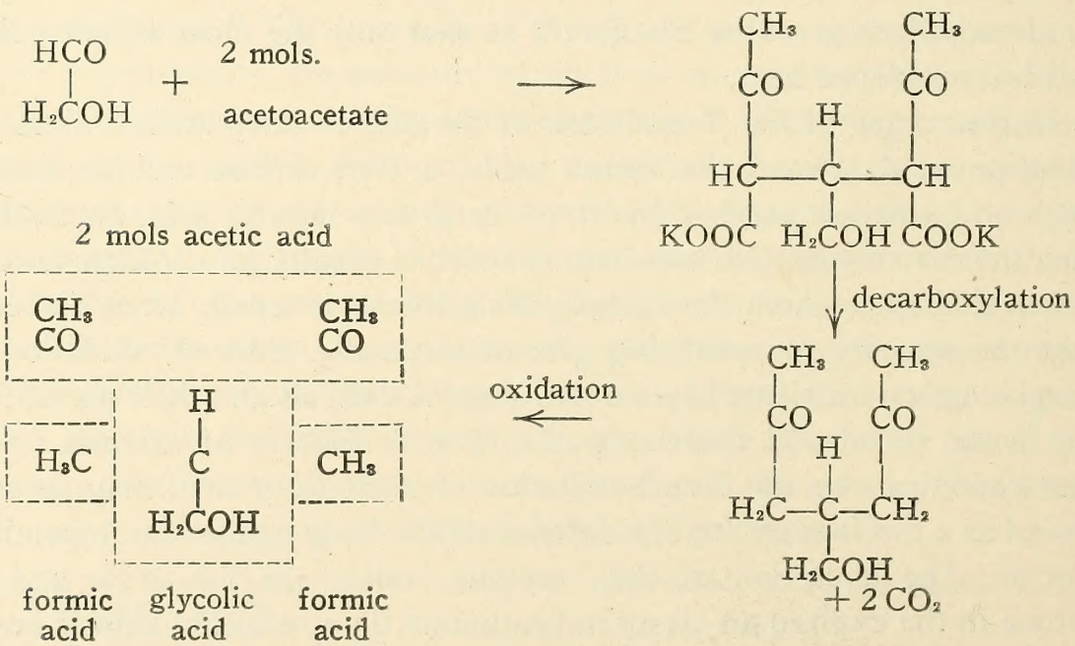
This apparently sharp differentiation of α-amino acids into possible sugar formers, on the one hand, and possible acetoacetic-acid formers, on the other, brings protein metabolism into prominence from the standpoint of a rather recent viewpoint of general metabolism that has been developed largely by the theoretical and experimental contributions of Shaffer. This viewpoint is based upon the observed fact that those nutrients normally yielding acetoacetic acid (or hydroxy-butyric acid) in the course of their catabolism cannot be oxidized beyond this stage unless glucose is simultaneously undergoing oxidation. Its most practical objective is the determination of the critical proportion between the nutrients undergoing metabolism at which "ketosis," *i.e.,* the accumulation of "acetone bodies" in the blood and their excretion in the urine, first appears. Its greatest significance obviously resides in the guidance it affords to dietetic control in diabetes, the main problem of which is the avoidance of ketosis, on the one hand, and of an undue strain on the carbohydrate tolerance, on the other. An excellent review of the subject has already been given by Shaffer,[101] so that only the most salient points need be considered here.

In the course of the β-oxidation of the higher fatty acids and of the oxidation of a few of the amino acids, a four-carbon residue results which on oxidation yields β-hydroxy- or β-keto-butyric acid. In fasting, when the carbohydrate metabolism is reduced greatly, or on high fat and protein diets, on which the carbohydrate intake is small, or in diabetes, when the capacity for oxidizing glucose is greatly reduced, oxidation of these ketogenic nutrients beyond this stage is difficult and their accumulation in the blood and tissues results. The formation of acetone under these conditions by the decarboxylation of acetoacetic acid may be considered as a mechanism for the defense of the body against an impending acidosis. The excretion of the "acetone bodies" in the urine and of acetone in the expired air, is an indication of the prevailing ketosis and a measure of its severity. According to the prevailing nomenclature, fatty

[101] Shaffer, P. A., "The Harvey Society Lectures," 1923-24, 105.

acids and those amino acids capable of yielding acetoacetic acid, are "ketogenic" factors, while carbohydrates, the glycogenic amino acids, and the glycerol of fats are "antiketogenic" factors. When the "ketogenic-antiketogenic balance" (ratio) increases to a certain critical value ketosis results.

The facts thus far stated point clearly to a definite chemical reaction between some oxidation product of glucose and acetoacetic acid, resulting in the formation of a compound much more readily oxidized than acetoacetic acid itself. This "ketolytic" reaction has been the subject of considerable research, particularly in Shaffer's laboratory. The first definite indication of such a reaction *in vitro* was obtained in 1921,[102] when it was shown that acetoacetic acid in alkaline solution and in the presence of hydrogen peroxide may resist oxidation for more than twenty-four hours, while in the presence of an excess of glucose it may be entirely destroyed in one hour or less at room temperatures. In later investigations with Friedemann,[103] Shaffer showed that glycol aldehyde, glyceric aldehyde, and other possible aldehyde decomposition products of glucose in the animal body also readily accelerate the oxidation of acetoacetic acid by peroxide in alkaline solution. Such results support the conclusion that the condensation of acetoacetic acid with the antiketogenic molecule proceeds as a Knoevenagel type of reaction, in which an aldehyde or ketone condenses with a 1,3-diketone or ketonic ester. Taking the case of glycol aldehyde, merely as an example, the ketolytic reaction is pictured as follows:



[102] Shaffer, P. A., *J. Biol. Chem.*, 1921, xlvii, 433.
[103] Shaffer, P. A., and Friedemann, T. E., *J. Biol. Chem.*, 1924, lxi, 585.

Reactions of this type may be catalyzed by certain amino acids and peptones, according to the work of Dakin.[104]

West [105] has further shown that glucose itself is capable of condensing with acetoacetic ester and that the condensation product reduces Fehling's solution in the cold within a few seconds and decolorizes weakly alkaline solutions of methylene blue in the cold. It is accordingly a more powerful reducing agent than glucose itself. Glucose itself, therefore, may be the ketolytic substance. Quoting Shaffer and Friedemann,

> "If this view proves to be correct for the antiketogenic action of glucose, it would indicate that the same oxidizing mechanism is concerned in the body both with glucose oxidation and the oxidation of its condensation product with acetoacetate, for both fail to occur in severe diabetes and both are restored by insulin. In the test-tube, alkali and hydrogen peroxide accomplish the oxidation of both simultaneously. These thoughts suggest the importance of the ketolytic reaction, though other means must be found to judge its direct applicability to physiological phenomena."

On the basis of these theoretical considerations, it appears that the ketolytic molecule, whatever its nature may be, condenses with two molecules of acetoacetic acid, if the latter be present in excess, the product being completely oxidized to carbon dioxide and water.

If antiketogenesis *in vivo* is the result of such a definite chemical reaction, it would be expected that as long as glucose is present in the metabolizing mixture in excess relative to the acetoacetic acid, all of the latter would disappear as fast as formed and no ketosis would result. Whenever there is insufficient glucose present to react with the acetoacetic acid as rapidly as it forms, ketosis and ketonuria would result, the amount of acetone bodies excreted being proportional to the glucose deficit. If the *in vitro* ketolytic reaction represents in the main the *in vivo* reaction, one would expect the critical molecular ratio between ketogenic and antiketogenic factors, at which ketosis appears, to be 2 to 1.

To test out these ideas, some method of estimating the ketogenic-antiketogenic balance must be devised. While several methods have been proposed for doing this,[106] they are all professedly merely approximations and are based upon essentially the same principles, so that, in continuing the consideration of Shaffer's own work and of that of his associates, a representative picture of current thought on this subject will be obtained. The method of Shaffer in its current form [107] may be briefly described as follows:

[104] Dakin, H. D., *J. Biol. Chem.*, 1909-10, vii, 49.
[105] West, E. S., *J. Biol. Chem.*, 1925, lxvi, 63.
[106] Ladd, W. S., and Palmer, W. W., *Proc. Soc. Exper. Biol. Med.*, 1920-21, xviii, 109; *Amer. J. Med. Sci.*, 1923, clxvi, 157. Woodyatt, R. T., *Arch. Intern. Med.*, 1921, xxviii, 125; "Endocrinology and Metabolism," New York and London, 1922, iv, 278.
[107] Shaffer, P. A., *J. Biol. Chem.*, 1922, liv, 399.

Ketogenic Equivalents in Millimols of Acetoacetic Acid.

Protein .....(a) grams urine N × 15

Fat .....(b) grams fat burned $\times \frac{3 \times 1000}{874}$ = grams fat × 3.43

Glucose Equivalents in Millimols.

Carbohydrate ..... (c) grams glucose $\times \frac{1000}{180}$ = grams glucose × 5.56

Protein .....(d) grams urine N $\times \frac{3.6 \times 1000}{180}$ = grams urine N × 20

Fat .....(e) grams fat burned $\times \frac{1 \times 1000}{874 \times 2}$ = grams fat × 0.57

$$\text{Ketogenic Ratio} = \frac{\text{total ketogenic mols}}{\text{total mols of glucose}} = \frac{a + b}{c + d + e}$$

Ketogenic Balance = total ketogenic mols — (2 × total glucose mols) = $(a + b) - 2(c + d + e)$.

Each molecule of fatty acid catabolized is assumed to give rise to one molecule of acetoacetic acid. Each molecule of leucine, phenylalanine, and tyrosine obtained from protein is also assumed to give rise to one molecule of acetoacetic acid. The value 874 is taken as the average molecular weight of mixed food or body fat, while the acetoacetic-acid-forming amino acids relative to total protein nitrogen were estimated on the basis of the composition of muscle proteins. This estimate gives a ratio of 10 millimols of acetoacetic acid to one gram of urinary N, but the estimate was arbitrarily raised from 10 to 15 millimols with the frankly expressed purpose of securing better agreement between calculations and theoretical expectations.

The antiketogenic factors are glucose as such, the glycogenic amino acids calculated from the extra-glucose to urinary nitrogen ratio (3.6 to 1) obtained in total diabetes, and the glucose derivable from the glycerol constituent of fats. It is interesting to note that protein is antiketogenic in its aggregate influence on metabolism.

The calculations are preferably based upon the analysis of metabolism from the respiratory exchange by the method of Zuntz and Schumberg, or less satisfactorily from the composition of the diet when fed in maintenance or submaintenance quantities.

From many analyses of the results of metabolism studies on diabetics and on fasting subjects according to the above method (or its equivalent for all practical purposes) Shaffer and many other investigators [108] have found that the threshold of ketosis in human subjects is represented by a ketogenic ratio of 1 : 1, rather than the ratio of 2 : 1 expected from *in*

[108] Richardson, H. B., and Ladd, W. S., *J. Biol. Chem.*, 1924, lviii, 931. Other references on the same point will be found in this article.

vitro experiments. The composition of diets that produce a ketogenic ratio of 1:1 is given by Shaffer as, "at least 10 per cent of the total calorie *requirement* as carbohydrate, about 10 per cent as protein and not more than 80 per cent as fat." It was further shown by Shaffer [109] that the threshold of ketosis was represented by a respiratory quotient of 0.76, which characterizes the combustion of a mixture of nutrients containing approximately equimolecular quantities of ketogenic and antiketogenic materials.

However, in subjects showing only slight ketosis, with ketogenic ratios less than 2:1, the excretion of acetoacetic acid was generally much less than would be expected from the calculated ketogenic balance based on a 1:1 ratio for the ketolytic reaction. On the other hand, in severe ketosis, it was found that the amount of ketonic acid excreted in many cases corresponds fairly closely with the calculated expectation based upon a ratio of 2 moles of acetoacetic acid destroyed for 1 mole of glucose burned, the same ratio indicated by the *in vitro* reaction. In other words, "when the metabolic mixture is such as to yield a very large excess of ketogenic molecules over the number of glucose molecules burned, the amount of total keto acid which is excreted approximately corresponds to the excess over and above *twice* the total glucose molecules burned." The calculations cited by Shaffer in support of this conclusion [107] are not, however, particularly convincing, though whether the frequent disagreement found between expectation and observation is due to the necessity of estimating (in many cases, merely guessing) the heat production of the subjects per 24 hours, or is due to a serious error in the principles upon which the expected results were calculated, cannot be judged at present.*

The fact that ketosis may appear before the ketogenic ratio reaches 2 to 1 is ingeniously explained by Shaffer [101, p. 125] as follows:

"When a large surplus of keto acid is not being formed it would be expected that some glucose molecules would be oxidized without encountering and reacting with keto acid, and thus the ketolytic value would be wasted; while at other localities there might be a deficit of glucose with consequent accumulation of keto acid. Such a condition exists, we may imagine, near the threshold of ketosis, when, due to uneven distribution and uneven metabolism in different parts of the body, considerably more than the theoretical minimum of glucose is necessary to avoid ketosis.

[109] Shaffer, P. A., *J. Biol. Chem.*, 1921, xlix, 143.

* It should be noted that the tabulation of such results given in his Harvey Lecture on "Antiketogenesis" [101] does not include all of the calculations Shaffer has made, but only the most successful ones, several of which are evidently the result of very fortunate guesses of the 24 hour heat production. The importance of the 24 hour heat production in the calculations rests in the fact that it forms the basis for the estimate of the amount of fat catabolized, an estimate profoundly affecting the computed ketogenic balance.

According to this view one might expect that the amount of the excess required to avoid ketosis would vary from the theoretical minimum to a considerably higher value. This expectation is borne out by the facts. Many subjects show slight ketosis with a metabolic ratio of 1 ketogenic to 1 total glucose, while considerably higher ratios may exist without very marked ketosis. Wilder and Winter have emphasized this fact. As a rule, however, the higher the ratio above 1:1 the more pronounced the ketosis until at 2:1 or above the amounts of total hydroxybutyric acid excreted are large and approximate more and more to the excess of keto acid over glucose calculated on a 2:1 ratio. The range between ratios of 1:1 and 2:1 is variable in that either very small or moderate ketosis may result."

In properly evaluating the antiketogenic theory, one should not be unduly discouraged by the frequent failures in its quantitative application to metabolic data. Rather its frequent successes in this direction should be given the greater weight, because of the necessary uncertainty in most of the factors used. It may legitimately be questioned, however, whether the factors for protein and fat should be based upon the composition of body protein and body fat, except in the case of fasting subjects. With subjects receiving high fat and high protein diets, as in diabetes, the character of the *dietary* fat and protein will determine their ketogenic or antiketogenic effect. For example, Janney [110] has shown that different proteins produce yields of glucose in phlorhizinized animals varying from 50 to 80 per cent by weight.

From a broad point of view, it seems a fair judgment that the essential features of the antiketogenic theory are correct. It accounts in a definite and reasonable fashion for an observation noted many years ago, and embodied in the frequently quoted expression of Rosenfeld that "fats burn only in the fire of carbohydrates." It accords with experimentally determined facts as satisfactorily as can reasonably be expected in view of the very inadequate means available of expressing it in a quantitative manner, though it is to be hoped that some experiments planned specifically to test the theory by obtaining *all* the data required will be undertaken in the near future.* The most practical application

[110] Janney, N. W., *J. Biol. Chem.*, 1915, xx, 321; *Arch. Intern. Med.*, 1916, xviii, 584.

* In this connection the successful experience of Osborne and Mendel [96] in inducing considerable growth in rats on carbohydrate-free rations containing as high as 75 per cent of fat is of interest, since the ketogenic ratios computed from the composition of the diets would lead one to expect considerable ketosis. However, the ketogenic ratio computed from a ration is of uncertain significance as an index to metabolism when the ration is consumed in amounts greatly in excess of the maintenance requirement

In this connection also the following quotation from Friedemann (*Proc. Soc. Exp. Biol. Med.*, 1926, xxiv, 223) is of interest:

"In sharp contrast to human beings most animals (dog, cat, pig, steer, goat, guinea pig, rat, rabbit, etc.) appear to develop only slight ketosis when starved. In man, on the other hand, starvation ketosis is a regular phenomenon, and is apparently a necessary consequence of the failure to metabolize a sufficient amount of carbohydrate. And conversely it is believed that the

of the theory is in putting the dietetic control of diabetics on a rational basis, and it seems to be serving this purpose well.

There is some danger, however, that the theory is being given too inclusive a significance with reference to glucogenesis from amino acids under normal conditions. The proof that certain amino acids are convertible into glucose, or that, under certain critical conditions characterized by an intensified demand for glucose, they are so converted in the animal body, is not equivalent to the proof that glucose is an *obligate* metabolite of the glucogenetic amino acids. It is quite conceivable that under less critical conditions no conversion to glucose occurs, particularly since such a conversion involves condensations of 2-carbon or 3-carbon compounds, representing an interruption in catabolism rather than a phase of catabolism.

THE SPARING EFFECT OF CARBOHYDRATE ON PROTEIN CATABOLISM

It is an opinion very generally held that the three main nutrients are replaceable in catabolism in rather definite amounts, their so-called "isodynamic" equivalents. The generalization has proven very useful in

metabolism of carbohydrate is essential for the normal complete combustion of the precursors of the acetone bodies. This seems applicable only to man. The ability to avoid ketosis without the direct intervention of metabolizing glucose appears to be possessed by most animals. Man is the exception. That animals have high tolerance for the acetone bodies is indicated by the results of acetoacetic acid injection [dog (Wilder, *J. Biol. Chem.*, 1917, xxxi ,59; Friedemann, Somogyi and Webb, *J. Biol. Chem.*, 1926, lxvii, 44), mouse (Robertson, *Med. J. Australia*, 1923, ii, 191), cat (Burn, *J. Physiol.*, 1925, lx, 16)]. This ability of animals to burn acetone bodies (apparently without the antiketogenic action of carbohydrate) would seem to be not an acquired property, but rather a function normal to all animals with the exception of man in whom it appears to have been lost. A parallel case is perhaps the ability to further metabolize uric acid to allantoin, possessed in general by mammalia, but lacked by man and the chimpanzee. The following observation has been made on a fasting monkey, which developed a ketosis quite comparable to that of man which was promptly abolished by glucose and food. Whether the results are representative of the behavior of the species or of primates generally can be determined only after observations on other individuals, the writer sees no reason to suppose that the behavior of this monkey is exceptional. The subject was an adult male brown capuchin monkey, whose probable normal weight was 1.3 to 1.4 kilos. He was sent to the laboratory suffering with "cage paralysis." The animal was placed in a small metabolism crate in a room kept at a temperature of about 30° C. The animal always sat at one end of the cage and, except for motions of the head, was very quiet. The absence of activity permits an approximate estimate of the total metabolism. The starvation was continued through the third day during which urine was collected in short periods without catheterization. At the end of three days glucose and food was given. The ketosis disappeared almost immediately. An increase in weight from 830 gms. to 1020 gms. occurred in less than 24 hours after consumption of food.

"When compared on the basis of body weight the ketosis of the monkey per kilo is more than 3 times as great as that of man. A more logical basis of comparison is on the basis of surface area or of total metabolism. The surface area was determined after death by two methods which agreed within 6 per cent. Measurements on various parts of the body and calculation of the surface area yielded a value of 0.143 square meter. The skin when stretched out on paper covered an area of 0.152 square meter. The calculations of the total metabolism were based on the following assumptions:

Surface area	0.152 sq. meter
Basal metabolism per square meter.....	40 Cal. per hour
Allowance for activity.....	10 per cent

This gives 160 Calories per 24 hours. The acetone body excretion per 1000 Calories heat output is of the same order of magnitude as that of man. It is of interest to attempt an evaluation of the acetone body excretion on the basis of the ketogenic versus anti-ketogenic factors used by Shaffer (*J. Biol. Chem.*, 1922, liv, 399). The expected or estimated excretion agrees remarkably well with the values found by analysis. This close agreement is consistent with the view that the marked ketosis in this animal was the consequence of the same factors which determine human ketosis." See also a later note by Friedemann in the 1927 *Proc. Am. Soc. Biol. Chem.*, lxi.

dietetics and in the rationing of farm animals and it must, therefore, be essentially correct, at least under practical conditions of feeding. However, the consideration of the ketogenic-antiketogenic balance just concluded shows that the equivalence does not hold beyond a certain critical concentration of carbohydrate in the diet, while nitrogen metabolism studies have shown that protein catabolism is affected to a different extent by carbohydrates and fats. This constitutes another exception to the "isodynamic law" of Rubner.

The difference in the effects of carbohydrates and of fats on protein catabolism is well illustrated by experiments on fasting animals. E. Voit [111] has shown very clearly that the intensity of protein catabolism in starvation is closely dependent upon the relative quantity of fat in the body, varying inversely with it. Evidently in the energy exchanges during total abstention from food, the fat stores in the body spare the nitrogenous tissues from disintegration in rather direct proportion to their relative size. However, C. Voit [112] found that if fat, even in considerable quantities, be given to a fasting animal, no appreciable diminution of the protein metabolism occurred, a result substantially confirmed by Bartmann.[113] Apparently the fat ingested simply spared body fat, exerting no considerable influence on protein metabolism. On the other hand, if a carbohydrate diet, or one predominantly carbohydrate, be given to a fasting animal, the protein catabolism as measured by the urinary excretion of nitrogen may be reduced to one-third of its former value.[114] Furthermore, the change from a predominantly carbohydrate diet to a fat diet of equal calorific value seems to be invariably accompanied by a marked increase in the excretion of urinary nitrogen. This is well shown by the following data cited by Cathcart: [115]

The Excretion of Urinary Nitrogen on Carbohydrate and Fat Diets.
(*All weights in grams.*)

Carbohydrate						Fat					
		Cathcart						Cathcart			
Landergren		I		II		Landergren		I		II	
Day	Urine N	Day	Urine N	Day	Urine N	Day	Urine N	Day	Urine N	Day	Urine N
1	8.91	1	6.40			5	4.28	5	4.83	3	5.25
2	5.15	2	4.77			6	8.86	6	8.13	4	9.01
3	4.30	3	4.79	1	8.12	7	9.64			5	13.30
4	3.76	4	4.39	2	6.65						

The sparing effect of carbohydrates on protein catabolism seems to be a general phenomenon, which is manifested at all levels of protein

[111] Voit, E., *Z. Biol.*, 1901, xli, 520, 545.
[112] Voit, C., "Physiologie des Stoffwechsels und der Ernährung," 1881, p. 128.
[113] Bartmann, A., *Z. Biol.*, 1912, lviii, 375.
[114] Landergren, E., *Skand. Arch. Physiol.*, 1903, xiv, 112. Cathcart, E. P., *Biochem. J.*, 1907, vi, 109.
[115] Cathcart, E. P., The Physiology of Protein Metabolism, London and New York, 1921, Chap. x, p. 144. See also Cathcart, E. P., *Biochem. J.*, 1922, xvi, 747.

nutrition in the sense that each addition of carbohydrate to a protein-containing diet will tend to lower the excretion of urinary nitrogen and thus increase the utilization of protein in anabolism. The increase in the biological value of protein, or in the growth secured per gram of protein consumed, with decreasing percentages of protein in predominantly carbohydrate diets reported by Mitchell [116] and by Osborne, Mendel and Ferry [117] may be so interpreted. Of the same significance is the observation of Folin and Berglund [68] that the ingestion of glucose by a human subject notably depressed the post absorptive level of amino acids, urea, undetermined nitrogen, and total non-protein nitrogen in the blood plasma.

In contradiction of almost every other investigator concerned with the relative sparing effects of carbohydrates and fats on protein metabolism, Maignon [118] has obtained results on rats indicating a markedly greater effect of fats than of carbohydrates. For example, a ration composed of equal parts of protein and fat (plus mineral salts) was capable of supporting constant weight in rats when consumed in quantities containing only 78 per cent of the energy of equally effective quantities of a ration made up of equal parts of protein and carbohydrate. The results are explained on the basis of a more intimate relation between the intermediary metabolism of fats and proteins than of carbohydrates and proteins. It is difficult to evaluate this work in the absence of a complete report of the details of the experiments and of the results secured. The rations used were obviously incomplete with respect to vitamins, but whether this fact is sufficient to explain the unique experimental results is uncertain. Until they are confirmed, however, by adequate experimental methods, they should perhaps be considered as unexplainable curiosities.

Various explanations have been given for the sparing effect of carbohydrates on protein catabolism. It has been associated by Kocher [119] with the similarity in their intermediate metabolites between glucose and at least the lower members of the amino acid series, the conclusion being that the ingestion of glucose-yielding nutrients leads to a "fixing of catabolized nitrogen by the dissociation products of glucose to form new proteins." This seems extremely unlikely, since the similarity only extends to alanine and possibly glycine, the resynthesis of which could hardly determine a synthesis of protein. Cathcart [115] suggests a relation

[116] Mitchell, H. H., *J. Biol. Chem.*, 1924, lviii, 905.
[117] Osborne, T. B., Mendel, L. B., and Ferry, E. C., *J. Biol. Chem.*, 1919, xxxvii, 223. *See also* Osborne and Mendel, *ibid.*, 1920, xli, 275.
[118] Maignon, F., *Compt. rend. Acad. Sci.*, 1918, clxvi, 1008; *ibid.*, 1918, clxvii, 172, 281; *ibid.*, 1919, clxviii, 474, 626. *Compt. rend. soc. biol.*, 1913, lxxii, 1054; *ibid.*, 1919, lxxxii, 398.
[119] Kocher, R. A., *J. Biol. Chem.*, 1916, xxv, 571.

between the antiketogenic action of carbohydrates and their protein-sparing effect, on the assumption that ketosis may in itself occasion an increase in protein catabolism. This explanation would, of course, only apply to the protein-sparing action of carbohydrates in conditions involving ketosis, such as protracted fasting. Landergren [114] offers an interesting and apparently probable theory. He believes that when the body is not receiving an adequate amount of glucose for the maintenance of the normal sugar content of the blood, it must manufacture it from other nutrients available. Since a physiological transformation of fat into sugar has never been satisfactorily demonstrated, he assumes that protein is the sole available source of glucose. The giving of carbohydrate in such a condition would spare that amount of protein previously serving as a source of blood sugar, while the giving of fat would not. In confirmation of this theory, Ringer [120] reports that the giving of small quantities of glucose to phlorhizinized animals results in a diminution in the excretion of urinary nitrogen, although all of the glucose administered is excreted in the urine. The finding by Nash [121] that the administration of insulin in phlorhizin diabetes also lowers the protein catabolism is given a similar interpretation. This theory also would only apply to conditions of very low carbohydrate intake.

A very reasonable explanation of the protein sparing effect of carbohydrate *at all levels of intake* would seem to follow merely from the known fact of the greater oxidizability of glucose than of any other nutrient. It is known that the glycogen stores in the body are much more readily depleted than the fat stores, and that a sudden muscular effort may be performed entirely at the expense of glycogen,[122] so easily is it oxidized by living cells. Bacteria will not attack protein or protein derivatives to an appreciable extent for energy production in the presence of glucose, while *in vitro* experiments amply testify to the relatively high "reduction potential" of sugar. The cells are being continually bathed by fluids containing amino acids, glucose, and fatty acids in various combinations. The extent to which these different nutrients will be drawn into the oxidative processes of the cells may depend more upon their relative reduction potentials or upon their physico-chemical state than upon their relative concentrations. An influx of glucose with a higher reduction potential than that of amino acids would obviously exert a marked sparing effect upon the later. Unlike glucose, fatty acids reach the tissues in the form of compounds held in mechanical suspension (emulsion) or in colloidal solution. It seems reasonable to suppose, there-

[120] Ringer, A. I., *J. Biol. Chem.*, 1912, xii, 431.
[121] Nash, T. P., *J. Biol. Chem.*, 1923, lviii, 453.
[122] Hill, A. V., *Science*, 1924, lx, 505.

fore, that they would be less readily accessible to oxidizing agents than compounds, such as glucose and amino acids, in true (molecular) solution, and hence less readily oxidized. An influx of fatty acids, therefore, might be supposed to exert a comparatively feeble "sparing" effect on amino acids. These theoretical considerations are offered to supplement, rather than displace, the theories explained above relating to the same general question, but applicable only under critical conditions of low carbohydrate supply or low carbohydrate tolerance.

THE TRANSFORMATION OF PROTEINS INTO FATS

Of all the possible transformations of nutrients in the animal body, that of the conversion of protein into fat, or, more correctly, of amino acids into the higher fatty acids entering into the composition of animal fats, has probably attracted the greatest amount of experimental enquiry and occasioned the most controversy. Perhaps this is due, on the one hand, to the fact that the production of sugar from protein and the production of fat from sugar were both rather easily demonstrable and early demonstrated, encouraging strongly a belief in the *possibility* of a conversion of protein to fat, while, on the other hand, the demonstration of a direct conversion of protein to fat has proven to be beset by many experimental difficulties. In the absence of a clear-cut demonstration of a direct conversion, several writers have been content in the past to assume the possibility of an indirect conversion through glucose. It may be questioned whether, under physiological conditions, such an indirect conversion occurs. The production of glucose from amino acids may be the result of two conditions, one an imperative need of the tissues for glucose as such; the other, the presence in the tissues of an excess of amino acids requiring immediate disposal. In the first case, the further conversion of glucose to fat would defeat the very purpose of its production from amino acids; in the second case, the natural course would seem to be the synthesis of glycogen rather than of fat. If the glycogen stores are already loaded to capacity, it is quite possible that the synthesis of glucose from amino acids would be effectively blocked in the absence of a ready means for the disposal of the glucose formed. In other words, it is conceivable that the conversion of glucose to fat may be forced by a large and persistent influx of glucose from the alimentary canal, but that the concentration of glucose in the tissues necessary to promote this conversion cannot be attained by the conversion of amino acids to glucose. Purely on chemical grounds, the conversion of protein to sugar to fat, involving a complicated and uneven series of cleavages, oxidations

and condensations, would seem to be a clumsy and thoroughly inefficient method of disposing of an excess of dietary protein.

A complete review of the literature dealing with the direct conversion of protein into fat need not be attempted here, not only because such reviews are already available,[123] but also because it is largely unprofitable except from an historical standpoint. The early work represents a dreary succession of investigations fatally marred by the failure to obtain *all* of the essential experimental data and the consequent necessity of assuming what should have been determined and, surprisingly, what could have been determined with little additional effort. One is forcibly struck by the quantity of controversial discussion excited by experimental data obtained upon one dog or one cat. It is perhaps justifiable, therefore, to discuss only those recent investigations that seem to be the most conclusive.

The production of fat from protein by bacteria seems to have been abundantly proved by the investigations of Beebe and Buxton [124] and of Ritchie.[125] That the lower forms of animal life can also accomplish the same conversion is clearly indicated by the experiments of Weinland [126] and of Nishikata [127] on the larvæ of certain species of flies, by the experiments of Hoffman [128] on the eggs of another species of flies and by the experiments of McClendon [129] on the eggs of the giant salamander and of the brook trout. The difficulties in demonstrating a conversion of protein to fat in the higher animals reside, first, in the necessity of excluding from the diet all considerable amounts of other nutrients that might be converted into or deposited as fat in the animal, namely, both carbohydrates and fats, and, second, in the fact that such high protein diets, even if consumed in large amounts, would, by their marked specific dynamic effects, set up conditions unfavorable to fat deposition. The experiments of Osborne and Mendel [130] on the continued growth of rats on rations containing 95 per cent of protein with no appreciable amounts of either carbohydrate or fat might have afforded a clear demonstration of fat formation from protein if the bodies of the rats had been analyzed. However, even in the absence of such analyses it seems extremely improbable that rats could grow from 40 to 80 grams in weight to 250 or even 336 grams, as they did in these experiments,

[123] Taylor, A. E., *Amer. J. Sci.*, 1899 cxvii, 569. Atkinson, H. V., *J. Metabolic Res.*, 1922, i, 565.
[124] Beebe, S. P., and Buxton, B. H., *Amer. J. Physiol.*, 1905, xii, 466. *See also* Walker, E. W. A., *Proc. Physiol. Soc., J. Physiol.*, 1922, lvi, xliv.
[125] Ritchie, W. T., *J. Path. Bacteriol.*, 1905, x, 334.
[126] Weinland, E., *Z. Biol.*, 1908, li, 197.
[127] Nishikata, T., *J. Biochem. (Japan)*, 1922, i, 261. *See Chem Abst.*, 1922, xvi, 4284.
[128] Hoffman, F., *Z. Biol.*, 1872, viii, 153.
[129] McClendon, J. F., *J. Biol. Chem.*, 1915, xxi, 269.
[130] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1924, lix, 13.

without simultaneously depositing considerable amounts of fat in their tissues.*

Recently the problem of fat formation from protein has been attacked in Lusk's laboratory by observing the respiratory exchange and the heat production of dogs after consuming large amounts of meat. In such an experiment reported by Williams, Riche, and Lusk [131] it was shown that during a period of 14 hours after the administration of 1200 grams of meat, much less carbon was eliminated in the respiration of the dog than corresponded to the total protein catabolism of the period as measured by the excretion of nitrogen in the urine. This fact indicates a retention of protein carbon. The quantity of carbon retained during the 14 hours was 13.8 grams. The actual amount of oxygen absorbed was 186.2 grams. If the protein carbon is assumed to have been retained as glycogen, the oxygen absorption would have been 184.5 grams, a difference of 0.9 per cent. If the protein carbon is assumed to have been retained as fat, the oxygen absorption would have been 169.7 grams, a difference of 10 per cent. Hence it is concluded that the former assumption is correct. The respiratory quotients obtained were generally appreciably less than 0.8, an observation agreeing with the conclusion of a conversion of protein to glucose. During these 14 hours, 34.5 grams of glucose are thus assumed to have been stored as glycogen in the organism. The urinary nitrogen eliminated amounted to 28.3 grams, the O : N ratio thus being 1.2 to 1, instead of the possible maximum of 3.6 to 1. It is concluded on these grounds that one-third of the glucose derivable from protein was retained in the organism and deposited in the tissues as glycogen, representing 20 per cent of the total energy contained in the protein catabolized.

In other experiments reported by Atkinson and Lusk,[132] the material retained after giving dogs 700 to 1300 grams of meat daily seemed to consist of mixtures of glycogen and fat possessing respiratory quotients varying from 0.77 to 0.96. In six of these experiments, the estimated R. Q. of the deposited material ranged from 0.83 to 0.86, representing a mixture of approximately 1 gram of fat to every 2 grams of glycogen. The calculations involved in these determinations are exemplified by the following figures relating to Experiment 55, and cover-

* However, Addis, McKay, and McKay (*J. Biol. Chem.*, 1926, lxxi, 139) found that rats raised on a diet containing 70 per cent of protein deposited very little fat in their bodies, and that for this reason such rats do not reach as heavy an adult weight as rats on diets containing ordinary percentages of protein. According to Addis, McKay, and McKay, the absence of fat in high-protein fed rats is evident during life, "but it is strikingly demonstrated when the abdominal cavity was opened, for instead of the heavy layer of postperitoneal fat seen in the control . . . rats only a small shrunken pad was left around the kidneys and elsewhere the bare muscles could be seen through the peritoneum."

[131] Williams, H. B., Riche, J. A., and Lusk, G., *J. Biol. Chem.*, 1912, xii, 349.

[132] Atkinson, H. V., and Lusk, G., *Proc. Nat. Acad. Sci.*, 1919, v, 247.

ing 5 hours of observation, during which the average excretion of nitrogen in the urine was 1.58 grams per hour:

	CO_2	O_2
Equivalent to 1.58 grams urinary N.....	14.77 grams	13.35 grams
Found in respiration per hour.....	11.42	10.42
Difference.....	3.35	2.93

On the assumption that protein was the only nutrient catabolized during this period, a subtraction of the observed respiratory exchange from that expected from the amount of protein deaminized (excretion of N in urine), gives a retention per hour of a pabulum that, if oxidized, would yield 3.35 grams of CO_2 and require 2.93 grams of O_2. The R. Q. of such a pabulum is 0.83, and the heat that would result from its oxidation is equal to 9.92 calories. Deducting this heat from that expected on the basis of complete oxidation of the protein deaminized, *i.e.*, 41.89 cals. (urine N x 26.5), gives 31.97 calories. This should represent the actual hourly heat production of the dog. The observed hourly heat production was 31.98 calories.

In still other experiments of Rapport and Lusk,[133] in which large amounts of meat were given and a carbohydrate meal was given the preceding evening in order to charge the glycogen reservoirs of the body, evidence was obtained for the retention of the carbonaceous residue of protein as fat only. In three experiments of this type, the average heat production per hour was 97.06 cals. by direct determination, 93.35 cals. by the ordinary indirect determination, 93.59 cals. by the method outlined in the preceding paragraph in which all of the protein carbon was assumed to have been retained as fat, and 103.16 cals. by the same method but in which all of the protein carbon was assumed to have been retained as glucose. The results evidently favor the conclusion drawn. A particularly favorable calculation is afforded from the results of Experiment 68:

Fifth Hour After Ingestion of 1000 Grams of Meat.

Urinary N = 1.44 grams.

	CO_2	O_2	Calories
Equivalent of 1.44 grams N.....	13.46 grams	12.17 grams	38.17
Found in respiration.....	10.10	8.72	
	3.36	3.45	11.32
Calories indirect			26.85
Calories direct			27.52

R. Q. of deposit = 0.708

[133] Atkinson, H. V., Rapport, D., and Lusk, G., *J. Biol. Chem.*, 1922, liii, 155.

Such calculations from the results of respiration experiments would be conclusive were it not for the uncertainty of some of the assumptions involved. The use of the excretion of urinary nitrogen as a measure of protein catabolism, while perhaps the best criterion available, cannot be accorded a high degree of accuracy, particularly when, as in these experiments, the rate of protein catabolism is varying hourly. The subtraction of the amounts of CO_2 and O_2 actually respired from the amounts calculated as equivalent to the urinary nitrogen must implicitly assume that the catabolism is all at the expense of protein. This assumption is entirely gratuitous and seems inherently improbable, since glucose in abundance must have been available for oxidation. And yet the calculations above illustrated would be meaningless unless this assumption is made. The agreement obtained between observed heat production and the heat production expected from these assumptions is frequently good, but it is well known that the calculation of total heat production by indirect calorimetry is not greatly affected even by large errors in the assumed proportions in which the different nutrients are being oxidized. For example, if the metabolism is all at the expense of glucose and the assumption is made that it is all at the expense of fat, the error in the calculated heat production would be only about 7 per cent, when made on the basis of the observed oxygen consumption; again, if the metabolism is all at the expense of protein and the heat production is calculated on the assumption of a non-protein metabolism, the error would amount only to a little over 6 per cent. The success of the method of indirect calorimetry used throughout Lusk's investigations is probably dependent upon the fortunate fact that the purely conventional theory of metabolism upon which it is based must be very greatly in error to occasion a considerable error in the calculated heat production. For these reasons, a good agreement between an experimentally observed heat production and one calculated on the basis of some particular assumption cannot be considered as a particularly strong confirmation of the accuracy of the assumption.

On the basis of these considerations, it is perhaps a fair judgment that a clear-cut demonstration of the production of fat from protein in the higher animals has not yet been reported, though a considerable probability that such a transformation occurs under certain conditions has been established.

THE DISPOSAL IN METABOLISM OF ABSORBED PRODUCTS OF INTESTINAL PUTREFACTION

By the action of bacteria upon proteins, protein derivatives, and amino acids in the large intestine, a series of fatty acids, hydroxy acids, alcohols, and amines are produced which are to some extent absorbed into the blood and are carried through the portal circulation to the systemic circulation. Skatole, indole, paracresol, phenol, putrescine, cadaverine, tyramine and histamine are perhaps the best known compounds of this nature. Many of these substances have such pronounced physiological effects that they may be considered truly toxic. The body, therefore, possesses a mechanism for detoxicating such compounds, a mechanism which seems to be largely located in the liver, if one may judge from the great susceptibility to "autointoxication" exhibited by animals with the Eck fistula.

The various chemical means by which such detoxication is effected has been admirably reviewed lately by Sherwin.[134] It is such a vast subject that more than passing comment can hardly be made here. The first method of detoxication would seem to be oxidation. If complete oxidation is impossible, and the partial oxidation effected does not adequately detoxicate, some other method is resorted to. Occasionally reduction, either independently or simultaneous with oxidation, is effected. If the putrefactive product resists these two methods effectively, it is next subjected to some synthetic type of reaction, in which it is combined with various organic compounds at the body's disposal to produce a non-toxic and more soluble substance. These synthetic reactions embrace conjugation with glycine, glutamine, or cysteine, and with sulfuric or glucuronic acids; methylation or acetylation or the formation of uramino acids has also been demonstrated experimentally. Even this ultimate method of detoxication may be only partially effective, so that a considerable proportion of the partially oxidized or partially reduced toxin may be excreted uncombined in the urine. According to Sherwin's findings it may be stated definitely[135] that:

"A given species of animal may have a certain definite mechanism of chemical defense against a given substance, while another species of animal may protect itself in an entirely different way. For instance, mammals, including man, protect themselves against ingested benzoic acid by joining or conjugating it with the amino acid glycocoll; but for the detoxication of the next acid in the series, phenylacetic acid, man is the only mammal whose organism conjugates it with the amino acid

[134] Sherwin, C. P., *Physiol. Revs.*, 1922, ii, 238.
[135] Novello, N. J., Wolf, W., and Sherwin, C. P., *Amer. J. Med. Sci.*, 1925, clxx, 888.

glutamin. All the lower animals, including the monkey, use glycocoll for this purpose." In contrast to animals, birds use ornithine in place of glycocoll in such conjugation detoxications.

As a result of bacterial action on nitrogen-containing substances in the large intestine, and of the chemical changes to which the bacterial products are subjected after absorption, a considerable number and variety of compounds have been found in traces in the urine of animals. The nature of these compounds is considered in some detail by Abderhalden.[136] Evidently they will vary in the amounts excreted with the intensity of the putrefactive processes occurring in the intestine, and in the proportions in which they occur in the urine with the kind of protein material consumed and the nature of the bacterial flora.[137] Many methods have been proposed for the quantitative determination of different groups of these compounds in the hope of obtaining a good quantitative expression for the extent of intestinal putrefaction, but such methods do not seem to be particularly successful,[135] not only from the chemical standpoint, but also because of the great variety of possible products and because the quantity of any one product excreted represents not the quantity produced, but something less than that, depending upon the resistance of the product to oxidation. In all probability, a study of the occurrence of these products in the blood is more profitable than a study of their occurrence in the urine.

Before leaving the subject of the manner in which the animal body deals with substances gaining access to its tissues that are of no food value and may therefore be considered as "foreign" bodies, it is extremely interesting to note the specific adaptation of animal metabolism to the chemical constitution of its normal food supply. The amino acids occurring in proteins are optically active, each amino acid occurring only as one of the two optical isomers. The other optical isomer in all cases is extremely resistant to catabolism; in fact, it is treated as a non-toxic foreign compound and is largely excreted in the urine unchanged. Another illustration of the same fact relates to the metabolism of the two aromatic amino acids, tyrosine and phenylalanine. The benzene ring in almost any other combination is most resistant to animal oxidation, while in these particular forms and in some of their derivatives, the benzene ring is readily broken and completely oxidized. In tyrosine, a hydroxyl group is present in the para position with reference to the side chain. Compounds identical with tyrosine, except that the hydroxyl group is in the ortho or meta position, are very slowly and incompletely

[136] Abderhalden, E., "Lehrbuch der Physiologischen Chemie," Berlin, 1923, 5 Auflage, I Teil, pp. 504-527.
[137] Underhill, F. P., and Simpson, G. E., *J. Biol. Chem.*, 1920, xliv, 69.

oxidized, the oxidation resulting, in so far as it proceeds at all, in the formation of *o*- or *m*-hydroxy-phenylacetic acid.[138] More illustrations of the same significance may be found in Sherwin's review.[134] It may possibly be true that the continuous excretion of amino acids in the urine, an excretion which seems to parallel the course of the exogenous metabolism of protein,[68] represents the excretion of a small fraction of amino acids or peptides that has been slightly changed by racemization during hydrolysis, or by slight bacterial action in the small intestine, and thus rendered resistant to animal oxidation.

[138] Blum, L., *Beitr. chem. Physiol. Path.*, 1907, xi, 142. Also, Flatow, L., *Z. physiol. Chem.*, 1910, lxiv, 367.

CHAPTER VII

SPECIAL PHASES OF AMINO ACID METABOLISM

The general transformations that all amino acids undergo in metabolism have been considered. However, many of the amino acids, possessing characteristic groupings, must undergo more or less individualistic metabolic changes, and for several of these amino acids it has been found possible to follow the unique phases of their metabolism to some extent, because of the availability of chemical methods for the detection or quantitative determination of their characteristic groups. These special phases of amino acid metabolism will now be considered.

ARGININE

Arginase

While many of the reactions that amino acids undergo in animal metabolism are presumed to be catalyzed by enzymes, the identification and characterization of such enzymes has never been successfully accomplished except in the case of arginase. Arginase, discovered by Kossel and Dakin [1] in 1904, is an enzyme concerned in the cleavage of arginine into urea and ornithine. The reaction is ordinarily written as follows:

$$\underset{\textit{Arginine}}{HN{:}C(N{H_2}).NH.CH_2.CH_2.CH_2.CHNH_2.COOH} + H_2O =$$

$$\underset{\textit{Urea}}{NH_2.CO.NH_2} + \underset{\textit{Ornithine}}{NH_2.CH_2.CH_2.CH_2.CHNH_2.COOH}$$

The enzyme was found to be present in the aqueous extract of the liver and to a considerably lesser extent in the kidney, thymus, lymph gland, and intestinal mucosa.

In later investigations of the distribution of arginase among animal organs, much contradictory evidence has been obtained. In mammals, the liver has always shown itself preëminent for its content of this enzyme,[2] while according to several investigators the liver of birds is without arginase activity. On the basis of such findings, Clementi [3] concludes

[1] Kossel, A., and Dakin, H. D., *Z. physiol. Chem.*, 1904, xli, 321.

[2] Fuchs, B., *Z. physiol. Chem.*, 1921, cxiv, 101; Edlbacher, S., *ibid.*, 1917, c, 111; Felix, K., and Tomita, M., *ibid.*, 1923, cxxviii, 40.

[3] Clementi, A., *Atti R. Acad. Lincei*, series 5, 1914, xxiii, 612; 1916, xxv, 366; 1918, xxvii, 299; 1922, xxxi, 559.

that arginase is present in the livers of those animals in which urea is the normal end-product of protein metabolism, but is absent from the livers of animals in which uric acid is the main protein metabolite. In this conclusion, he is supported by Hunter and Dauphinee,[4] but Edlbacher and Bonem [5] have apparently demonstrated a considerable arginase activity of the livers of cockerels, although the livers of female birds (hens and pigeons) showed none. The male pigeon resembled the female pigeon in this respect.

Concerning the occurrence of arginase in the kidney, it appears from most of the reported experiments that in mammalian kidneys it is absent or in slight concentration only, while in birds it occurs in much more considerable amounts in this organ. According to Hunter and Dauphinee, most fishes occupy an intermediate position in this respect. Edlbacher and Bonem have not found such a sharp distinction between mammals and birds, their results indicating a considerable arginase activity of the kidneys in both classes, though somewhat greater for birds.

In herring and dog fish, and possibly in all fishes, the organ next in activity is the heart,[4] while in other vertebrates—at least in mammals, birds and chelonian reptiles—the heart is inactive.

Concerning other organs than the liver and kidney, negative results have ordinarily been obtained with mammals, though Edlbacher and Bonem have reported a rather constant and considerable occurrence of arginase in testicles. Later work by Edlbacher and Röthler [6] emphasizes the sex difference in the occurrence of arginase. Among mammals as well as birds it was found that female organs contain only from 60 to 70 per cent of the arginase that was found in male organs, and that arginase occurs in testicles in amounts somewhat proportional to their maturity, while ovaries are free from this enzyme.

In the tissues of invertebrates, arginase has rarely, if ever, been found.[4] From their extensive investigations with what appears to be a well-controlled method, Hunter and Dauphinee suggest that the restriction of arginase in the higher vertebrates to one or at most two organs is a condition evolved from a primitive state, exemplified still in the dog fish and in a smaller degree in some other fishes, in which arginase is rather generally distributed throughout the body. The occurrence of arginase in bacteria has recently been reviewed and investigated by Kossel and Curtius.[7]

It is difficult to assess the significance of arginase in the metabolism

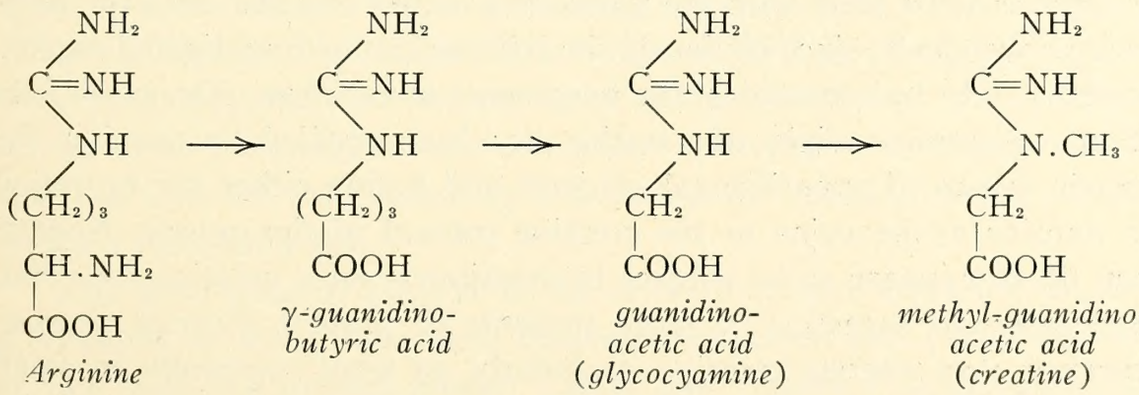
[4] Hunter, A., and Dauphinee, J. A., *Proc. Roy. Soc. London,* 1924-25, series B, xcvii, 227.
[5] Edlbacher, S., and Bonem, P., *Z. physiol. Chem.,* 1925, cxlv, 69.
[6] Edlbacher, S., and Röthler, H., *Z. physiol. Chem.,* 1925, cxlviii, 264, 273.
[7] Kossel, A., and Curtius, F., *Z. physiol. Chem.,* 1925, cxlviii, 283. *See also* Sendju, Y., *J. Biochem. (Japan),* 1925, v. 229.

of arginine. Felix and Tomita [2] believe that liver arginase is sufficiently active to destroy all the free arginine coming to the liver from the portal blood. It is impossible, however, to accept this view, if only because the advantage to the organism of such a wholesale destruction of an amino acid occurring to such a large extent in tissue proteins seems obscure. Furthermore, Gross [8] has shown that the reaction proceeds but slowly in dilute solution and possibly is not capable of proceeding to completion. Arginase undoubtedly initiates one pathway of arginine metabolism which is probably the pathway leading to sugar production. It will be recalled (p. 305) that of all the amino acids freely giving rise to sugar in phlorhizinized dogs, arginine is the only one containing more than 5 carbon atoms. In this case, in all probability, the sugar is being formed from ornithine, which is known to be glucogenetic. The predominant occurrence of arginase in the kidney, rather than the liver, of birds may possibly be associated with the use by these animals of ornithine rather than of glycine in the detoxication of benzoic acid (see p. 323).

Thomas [9] has shown that liver press juice will split γ-guanidinobutyric acid into urea and γ-amino-butyric acid, but will not attack ε-guanidino-caproic acid. The former cleavage is probably due to arginase.

The Relation Between Arginine and Creatine

In all probability, tissue creatine is derived from some one or more of the amino acids resulting from protein digestion. The amino acid related closest chemically to creatine is arginine, and according to Knoop and Neubauer, the transformation of the latter into the former may be reasonably pictured as follows:



The first step is the result of oxidative deamination, the second is the result of β-oxidation (Knoop), and the third step is the result of methylation. The first two steps are in all probability normal metabolic transformations of arginine in so far as it escapes the action of arginase, while

[8] Gross, R. E., *Z. physiol. Chem.*, 1920, cxii, 236.
[9] Thomas, K., *Z. physiol. Chem.*, 1913, lxxxviii, 465.

the last step, methylation of an amino (or substituted amino) group, is one that the body is known to be capable of performing, as exemplified by the occurrence of sarcosine (methyl glycocoll) in muscle, and by the conversion of pyridine into methyl-hydroxy-pyridine by various animals.[10]

While the theoretical possibility of the conversion of arginine into creatine is thus clear, its experimental demonstration has yielded as many negative as positive results, an eventuality that has tended to confuse the issue. It should, however, be realized that from the nature of the problem, the significance of a negative result is ambiguous, since it may represent merely an unsuccessful attempt to reproduce conditions favorable to creatine formation other than the presence of a suitable precursor. Since creatine is a tissue constituent with a special function, it is not to be expected that the rate of its formation is solely dependent upon the amount of precursor available, as would be the case if creatine were a waste product of amino acid catabolism, or an obligate intermediary product. On the other hand, the significance of a positive result, that is, a demonstration of creatine production in response to the administration of arginine, is clear and undivided, and cannot be discounted by failures to obtain such a response under other conditions. A positive result proves the possibility of the transformation and establishes arginine as a precursor of creatine, without, however, implying that there is no other precursor in the protein molecule.

The Exogenous Origin of Creatine

A study of the experiments concerned with the relation of arginine to creatine must start with the question whether creatine can ever originate exogenously, since obviously the relation can be investigated experimentally only by controlling the exogenous metabolism. The possibility of an exogenous origin of creatine has been studied by varying the protein intake of experimental subjects and noting either the excretion of creatine in the urine or the creatine content of the muscle. Normal men do not appear to be subject to creatinuria on a creatine-free diet, even after the ingestion of large amounts of protein. Normal women, however, may excrete creatine irregularly, or even constantly in small amounts.[11] This type of creatinuria is probably of endogenous origin, possibly due to a somewhat unstable endogenous catabolism, frequently accelerated by labile endocrine activity. It has been shown, also, that marked creatinuria may be induced in women by feeding large amounts

[10] Sherwin, C., *Physiol. Rev.*, 1922, ii, 272.

[11] Krause, R. A., *Quart. J. Exp. Physiol.*, 1911, iv, 293; Rose, M. S., *J. Biol. Chem.*, 1917, xxxii, 1; Stearns, G., and Lewis, H. B., *Amer. J. Physiol.*, 1921, lvi, 60.

of protein. Denis and Minot have demonstrated this fact in four experiments on women.[12] The creatinuria thus induced roughly paralleled the exogenous protein catabolism, and could be increased or diminished at will by suitable variations of the protein intake. Denis has also shown that in the case of hospital patients with creatinuria the quantity of creatine excreted during the night is but a fraction of that eliminated in the day,[13] confirming in this respect the observations of Powis and Raper.[14] In some cases creatine was entirely absent from the night urine, while present in considerable quantities in that passed during the day. Furthermore, the day's excretion was greatest after meals, when protein catabolism was greatest, a relation not observed by Powis and Raper. M. S. Rose,[11] in her investigations on creatinuria in women, did not observe any relation between the protein intake and the excretion of creatine; however, her subjects were probably not receiving as large amounts of protein as the subjects of Denis, and the absence of analyses for total urinary nitrogen precludes any comparison of the results of these two investigators. In patients with exophthalmic goitre, both male and female, Denis [15] has also found a relation between the protein intake and the amount of creatine excreted in the urine. Gibson and Martin [16] have observed the same relation in pseudo-hypertrophic muscular dystrophy.

The failure of Rose, Dimmitt, and Bartlett [17] and others [18] to confirm the findings of Denis as to the influence of high protein feeding on the creatinuria of women may be due to a fortuitous selection of subjects, or to the fact that the diets tested did not contain as large amounts of protein as those of Denis, or that the periods of observation were too short, or that they did not use gelatin as did Denis and Minot. Gelatin is richer in arginine than either the proteins of milk or of eggs. Whatever the explanation may be, the comment of Hunter [19] that "such failures do not destroy the significance of positive observations; but they emphasize the difficulty of producing creatinuria by protein feeding alone," seems a reasonable one.

The occurrence of creatine in the urine of infants and children, first discovered by Rose,[20] has been frequently reported and frequently correlated with the protein intake. Talbot and Gamble [21] investigated the

[12] Denis, W., and Minot, A. S., *J. Biol. Chem.*, 1917, xxxi, 561; 1919, xxxvii, 245.
[13] Denis, W., *J. Biol. Chem.*, 1917, xxix, 447.
[14] Powis, F., and Raper, H. S., *Biochem. J.*, 1916, x, 363.
[15] Denis, W., *J. Biol. Chem.*, 1917, xxx, 47.
[16] Gibson, R. B., and Martin, F. T., *J. Biol. Chem.*, 1921, xlix, 319.
[17] Rose, W. C., Dimmitt, J. S., and Bartlett, H. S., *J. Biol. Chem.*, 1918, xxxiv, 601.
[18] Stearns, G., and Lewis, H. B., *Amer. J. Physiol.*, 1921, lvi, 60.
[19] Hunter, A., *Physiol. Revs.*, 1922, ii, 610.
[20] Rose, W. C., *J. Biol. Chem.*, 1911, x, 265.
[21] Talbot, F. B., and Gamble, J. L., *Amer. J. Dis. Children*, 1916, xii, 333.

effect on the protein metabolism of an infant of increasing the protein intake from 10 to 37.4 grams per day, with a fairly constant caloric intake. While the creatinine remained at a level of 20 to 25 mgms. of nitrogen per day, the creatine nitrogen increased steadily from 1 to 40 mgms. Denis and Kramer,[22] likewise, have presented experimental results on four children and one infant, which indicate that the amount of creatine found in the urine of children is directly dependent on the intake of protein, being high when large quantities of protein are ingested, and low, or in some cases absent entirely, when the child is fed a diet of an extremely low protein content.

Gamble and Goldschmidt [23] have confirmed the results of Denis and Kramer on infants, but have related the varying excretion of creatine with the varying intake of milk whey rather than the varying intake of protein. On the basis of these results, they suggest that variations in creatinuria in infants obtained by feeding variable amounts of milk with the purpose of securing different intakes of protein may really be due to the creatine content of the whey, which Denis and Minot [24] have shown to be appreciable. This conclusion is fortified by the results of another investigation on infants [23] in which it was shown that ingested creatine may be practically completely recovered in the urine. However, the conclusion of Gamble and Goldschmidt does not appear to be well founded upon their own data. It seems to be based upon the results of 2 out of 5 experiments, and even in these two, assuming a quantitative excretion of ingested creatine, the variations in whey creatine could not account for more than 50 per cent of the variation in creatine elimination. Furthermore, an experiment on an infant, reported by Bosworth, Bowditch, and Ragle,[25] indicates no marked effect on the creatine eliminations of the amount of whey consumed.

It seems fair to conclude, therefore, that the excretion of creatine by infants and children is induced by the feeding of protein, though it may to some extent be of endogenous origin, since in some cases, even on an extremely low protein intake, considerable amounts of creatine may still be excreted.[23] The main difference between these subjects and adult woman, therefore, seems to be the relative ease with which children respond to even moderate variations in protein intake. In what way this may be correlated with the probable lower capacity to destroy creatine of the younger subjects, or with the lower concentration of creatine in

[22] Denis, W., and Kramer, J. G., *J. Biol. Chem.*, 1917, xxx, 189.
[23] Gamble, J. L., and Goldschmidt, S., *J. Biol. Chem.*, 1919, xl, 199, 215.
[24] Denis, W., and Minot, A. S., *J. Biol. Chem.*, 1919, xxxviii, 453.
[25] Bosworth, A. W., Bowditch, H. J., and Ragle, B. H., *Amer. J. Dis. Children*, 1915, ix, 120.

their tissues, is problematic. Any relation of the latter factor is not at all obvious.

In further confirmation of the conclusion that creatine may be of exogenous origin, the experiments of Mitchell, Nevens and Kendall [26] on rats may be cited. These investigators found that the concentration of creatine in the bodies of rats subsisting upon a protein-containing ration was distinctly higher than that of rats maintained for varying periods of time on a practically nitrogen-free ration. In the former rats, it may be assumed that both exogenous and endogenous catabolism of nitrogenous compounds were operating, while in the latter case the endogenous catabolism only (or mainly) was occurring. It seems fair to presume, therefore, that the reduction in the concentration of creatine was due to the absence of (or marked reduction in) an exogenous protein catabolism. It may be noted that no difference between the two groups of rats was observed in the concentration of total nitrogen, non-protein nitrogen, amino nitrogen, total sulfur, or non-protein sulfur of the total tissues.

The question may be raised, whether the creatine eliminated by children and women in response to an increased intake of protein, as well as the similar response by pigs demonstrated by McCollum and Steenbock,[27] is of exogenous or endogenous origin. It may be that the ingestion of large amounts of protein by these subjects, inducing an excessive exogenous catabolism, leads to the formation of creatine as an atypical end-product of the catabolism of some precursor in the protein molecule. This seems the most direct explanation of the facts observed. On the other hand, it may be argued that excessive exogenous protein metabolism may stimulate endogenous catabolism, as a result of which creatine appears in the urine, since creatine seems to be a typical end-product of an accelerated endogenous catabolism (see p. 474). If the former explanation is correct, it would be possible to get different effects with different proteins, depending upon their content of the creatine precursor. Steenbock and Gross,[27] and, earlier, McCollum and Steenbock, have noted such differences in the response of pigs to different proteins, edestin and flaxseed proteins being especially effective, casein less so, and the corn proteins still less effective. They suggest on the basis of other work than their own, that arginine is the creatine precursor in the protein molecule.

Hunter [19] believes that the demonstration that protein feeding may increase creatine excretion "is not necessarily to be interpreted as proving an exogenous source for creatine, in the sense that the latter may

[26] Mitchell, H. H., Nevens, W. B., and Kendall, F. E., *J. Biol. Chem.*, 1922, lii, 417.
[27] McCollum, E. V., and Steenbock, H., *J. Biol. Chem.*, 1912-13, xiii, 209. *Also* Steenbock, H., and Gross, E. G., *J. Biol. Chem.*, 1918, xxxvi, 265.

arise directly, like urea, from certain precursors in the ingested protein molecule. There may be another explanation of the phenomenon. It may represent merely one phase of that general stimulation of cellular metabolism which is described as the specific dynamic action of protein. An increased *endogenous* production of creatine, resulting from such stimulation, might be expected to manifest itself in just the sort of creatinuria that follows protein ingestion. It would be brought about only by catabolized protein, since protein deposited in the form of new tissue exerts no specific dynamic action; it would be at its height, as Denis and Kramer found it to be, during the second and third hours after ingestion, when heat production has reached its maximum but the output of urea is still rising; and it would occur most readily in those conditions where the metabolism is already relatively high, as in exophthalmic goiter, or in childhood. Such an explanation of the creatinuria following a high protein diet seems therefore to be well worthy of consideration. Protein feeding will increase also the output of uric acid; but no one has sought the origin of uric acid directly in the protein of the diet. Lewis, Dunn and Doisy [28] have shown reason to believe that proteins and amino-acids increase the production of endogenous uric acid by virtue of their general property of stimulating all cellular metabolism; it does not seem improbable that they should simultaneously increase the production of endogenous creatine."

The theory that the exogenous protein catabolism, by reason of its specific dynamic effect on the cells, stimulates the endogenous catabolism, receives no support from the creatinine excretion, which is unaffected by protein feeding. If this theory is correct, the independence between these two types of protein catabolism that is the basic and original contribution of Folin's theory is incorrect. The data of Lewis, Dunn and Doisy with reference to uric acid excretion are not themselves in good agreement with the theory that amino acids, by their specific dynamic action, stimulate the endogenous catabolism, since those amino acids possessing the greatest effect upon uric acid elimination, *i.e.*, glutamic and aspartic acid, have been shown by Lusk [29] to be without specific dynamic effect. It may, in fact, be argued that the data of Lewis, Dunn and Doisy are more easily explained by the old hypothesis of Mares that the origin of the increased amounts of endogenous uric acid following the ingestion of protein (and hence amino acids) is to be attributed

[28] Lewis, H. B., Dunn, M. S., and Doisy, E. A., *J. Biol. Chem.*, 1918, xxxvi, 9. However, the results of Lewis, Dunn, and Doisy have not been confirmed by H. Zwarenstein (*Biochem. J.*, 1928, xxii, 307). Under the conditions of his experiments, neither the ingestion of glycine or alanine, nor of a purine-free protein meal (egg white and cheese) affected the hourly output of uric acid or of creatinine.

[29] Lusk, G., *j. Biol. Chem.*, 1912, xiii, 155; Atkinson, H. V., and Lusk, G., *Ibid.*, 1918, xxxvi, 415.

mainly to the activity of the secretory glands of the gastrointestinal canal. Although the investigators themselves thought that this explanation could not be applied to amino acids administered as such, since they require no digestion, nevertheless it is quite possible that they might stimulate the gastric and intestinal glands. With respect to the gastric glands, Ivy and Javois [30] have demonstrated the stimulating effect of hydrolyzed proteins and individual amino acids. Hence, the data of Lewis, Dunn and Doisy are not in close agreement with the theory that proteins and amino acids, administered *per os,* stimulate the endogenous catabolism. That the creatinuria induced by protein feeding also is not brought about by reason of the specific dynamic action of this nutrient is indicated by the fact that different proteins have different effects on creatine excretion,[27] although possessing very similar specific dynamic effects.[31]

From these considerations it appears that the simplest explanation of the demonstrated relation of protein feeding and creatine excretion in certain subjects, *i.e.,* that creatine may be formed from dietary protein, is the more probable one. Since creatine may be formed from exogenous amino acids, it is next in order to inquire whether arginine is a possible precursor.

Arginine as a Precursor of Creatine

Undoubtedly the most probable precursor of creatine among the amino acids occurring in protein is arginine, likewise a guanidine derivative. A good deal of experimental work has been done to establish this connection, in the way of feeding and injecting arginine or some of its probable intermediate metabolites, or by perfusion and incubation experiments with animal tissues *in vitro.* The problem is evidently one in which positive results may be obtainable only under restricted (and unknown) conditions, since in adult animals especially it is evidently extremely difficult to force a formation of creatine. It seems fair to conclude, therefore, that negative results secured in this field are of no definite significance. In the face of positive evidence of a conversion of arginine to creatine, negative results may represent simply failures to impose necessary conditions, rather than demonstrations that the conversion cannot be made.

Glycocyamine (guanidine acetic acid), a probable metabolite of arginine, is convertible into creatine by methylation. Several experiments reported in the literature indicate the possibility of such a methylation in animal metabolism. In 1906, Jaffé [32] obtained increases in the excre-

[30] Ivy, A. C., and Javois, A. J., *Amer. J. Physiol.,* 1925, lxxi, 583, 591.
[31] Rapport, D., *J. Biol. Chem.,* 1924, lx, 497; Weiss, R., and Rapport, D., *Ibid.,* 513.
[32] Jaffé, M., *Z. physiol. Chem.,* 1906, xlviii, 430.

tion of creatine in the urine, and, less certainly, in the creatine content of the muscles, by the oral administration of glycocyamine to rabbits. Dorner,[33] in the following year, confirmed and supplemented Jaffé's results, using Folin's analytical methods for creatine and creatinine. The addition of glycocyamine to autolyzing muscle appeared to induce a formation of creatine, a result that was duplicated by Palladin and Wallenburger.[34] The latter investigators also reported increases in the creatine content of muscle following the subcutaneous injection of glycocyamine into rabbits.

Thompson [35] performed experiments upon dogs and birds in which glycocyamine was given *per os*. Distinct increases were obtained in the output of total urinary creatinine in the case of two dogs, and enormous increases in the case of four birds. The results do not appear to be appreciably vitiated by the possibility of the glycocyamine being excreted in the urine unchanged and, upon conversion to glycocyamidine by boiling with hydrochloric acid in the ordinary procedure of Folin for urinary creatine, reacting with the picric acid and alkali to give a red coloration itself. In two experiments upon rabbits, the intravenous injection of glycocyamine appears to have increased the creatine content of the muscle.

Baumann and Hines [36] have shown that the subcutaneous injection of glycocyamine in rabbits and dogs produced a significant increase in an existing creatinuria, though no effect upon the creatine content of muscle was observed. In one feeding experiment upon a man 23 years of age, excreting an average of 20 mgms. of creatine daily in the urine on a creatine-free low-protein diet, a marked increase in creatinuria occurred upon feeding glycocyamine. While this result is considerably discounted by the authors because of the presence of glycocyamine in the urine, a similar result was later obtained by Gibson and Martin [37] in feeding experiments on a 12-year-old boy suffering from progressive pseudohypertrophic dystrophy. Of 0.5 gram of glycocyamine given by mouth, at least 36 per cent was converted into creatine with no effect upon the creatinine excretion. In obtaining this figure a correction was made in the urinary analysis for the vitiating effect of the glycocyamine itself.

More recently Stuber and his associates [38] found that the intravenous injection of glycocyamine into rabbits and men caused an immediate increase in the creatine, and particularly the creatinine, in blood. The

[33] Dorner, G., *Z. physiol. Chem.*, 1907, lii, 225.
[34] Palladin, A. and Wallenburger, L., *Compt. rend. Soc. Biol.*, 1915, lxxviii, 111.
[35] Thompson, W. H., *J. Physiol.*, 1917, li, 111.
[36] Baumann, L., and Hines, H. M., *J. Biol. Chem.*, 1917, xxxi, 549.
[37] Gibson, R. B., and Martin, F. T., *J. Biol. Chem.*, 1921, xlix, 319.
[38] Stuber, B., Russman, A., and Proebsting, E. A., *Biochem. Z.*, 1923, cxliii, 221.

increase reached its height in about 5 minutes and usually disappeared in an hour. A further finding of great interest was that thyroidectomized rabbits gave no indication of a methylation of glycocyamine and that methylation could be induced in such animals by the feeding of thyroid substance, of iodine or iodides, and of normal blood, but not by the feeding of blood from thyroidectomized animals. The results are interpreted as indicating that the methylation of glycocyamine is a function of the thyroid gland, and that iodine is used by the body in methylating organic compounds in much the same way as it is used in the chemical laboratory.

In spite of the fact that negative results relative to the transformation of glycocyamine into creatine have been reported,[39] it seems clear that the transformation in the animal body has been abundantly demonstrated.

Thomas has reported negative results in a series of experiments that attempted to demonstrate some physiological relation between creatine and several other probable metabolites of arginine, as well as compounds which, upon β-oxidation, would yield a probable metabolite. In experiments involving enteral or parenteral administration of the compounds, or perfusion through muscles, Thomas was able to obtain no definite evidence of the conversion into creatine of ε-guanido-caproic,[40] ε-methyl-guanido-caproic, γ-guanidobutyric, or γ-methyl-guanidobutyric acids,[41] or of δ-methylarginine.[42] In commenting upon these results, Hunter [19] says:

"Among all the conceivable intermediates of an arginine-creatine transformation glycocyamine remains the only one which gives positive results; and there are difficulties in accepting it as a real intermediate of normal metabolism. It is for the most part excreted unchanged, only a fraction of the administered dose undergoing methylation; and although only slightly soluble, it has never been detected as a constituent of animal tissues."

The criterion here given of a normal intermediate is unnecessarily severe, and would doubtless rule out the large majority of compounds generally accepted as intermediate metabolites in protein, carbohydrate, and fat metabolism. Speaking of biological methods of determining the nature of intermediary metabolites Dakin [43] says, "the behavior of a substance when gradually produced, at low concentration and rapidly

[39] Czernecki, W., *Z. physiol. Chem.*, 1905, xliv, 294. *Also* Mellanby E., *J. Physiol.*, 1907-08, xxxvi, 447.
[40] Thomas, K., *Z. physiol. Chem.*, 1914, xcii, 163.
[41] Thomas, K., *Z. physiol. Chem.*, 1918-19, civ, 73.
[42] Thomas, K., *Ber. ges. Physiol.*, 1920, ii, 170.
[43] Dakin, H. D., "Oxidations and Reductions in the Animal Body," London, 2nd ed., 1922, p. 22.

undergoing further change, may be very different from that of the same substance when rapidly injected in relatively high concentration into the tissues of an animal. Indeed, in the latter case it is always uncertain whether the substance ever really reaches the sphere of action in the cells normally concerned with its metabolism." The same remarks undoubtedly apply to administration *per os*. Large losses of normal metabolites reaching the cells under these unnatural conditions are to be expected,[44] and such losses cannot reasonably be urged against the conclusion that a compound administered thus artificially is not a normal metabolite. The fact that glycocyamine has never been detected as a constituent of animal tissues * possesses no significance in this connection, and the argument of Hunter relative to it is reminiscent of the old theory of protein regeneration in the intestinal walls based upon the negative results of all attempts to demonstrate the presence of amino acids in the blood. The significance of negative experimental results is inconsiderable unless it can be shown that they represent something more than a failure to produce certain possible or expected effects because of the use of inadequate experimental methods or of the prevalence of unsuitable experimental conditions.

Direct attempts to secure a transformation of arginine into creatine in animal tissues have been unsuccessful in many experiments,[45] not always because possible effects were not observed, but because they were not considered significant in view of the existence of uncontrolled variable factors. For example, Baumann and Hines invariably observed increases in the excretion of creatine whenever arginine was injected, but because of the variable excretion of this metabolite the significance of the increases could not be established. They were reluctant to attach any significance to the arginine increases for another reason also. In one experiment an increase in creatine excretion followed an injection of histidine. This was considered as a control experiment, but if histidine is convertible into arginine, as Ackroyd and Hopkins believe, it cannot be so considered.

The most extensive experiments concerned with the relation of arginine to creatine have been reported by Thompson.[46] The care with which the experiments were planned, carried out, and controlled, as well as the positive character of the results obtained, warrant a somewhat

[44] Leiter, L., *J. Biol. Chem.*, 1925, lxiv, 125.

* The fact that there is no satisfactory method for determining glycocyamine and that apparently no attempt has been made to detect it in animal tissues, is perhaps pertinent to this discussion.

[45] Jaffé, M., *Z. physiol. Chem.*, 1906, xlviii, 430; Baumann, L., and Marker, J., *J. Biol. Chem.*, 1915, xxii, 49; Baumann, L., and Hines, H. M., *J. Biol. Chem.*, 1918, xxxv, 75; Thomas, K., *Ber. ges. Physiol.*, 1920, ii, 159; Rose W. C., and Cook, K. G., *J. Biol. Chem.*, 1925, lxiv, 325.

[46] Thompson, W. H., *J. Physiol.*, 1917, li, 111.

extended consideration of them, particularly in view of the casual way in which they are ordinarily referred to: the positive results obtained are considered as somewhat anomalous and considerably discounted by the negative results reported by other investigators. But where other investigators have made one or two observations upon one or two animals, Thompson has reported 73 observations upon a large number of dogs, birds and rabbits in 5 series of experiments, not including the control experiments or experiments not bearing directly upon the relation of arginine to creatine.

Thompson's results may be summarized as follows:

1. In experiments upon 4 dogs and 7 ducks it was found that the feeding of arginine carbonate resulted, in all cases but one, in an increased excretion of total creatinine in the urine in each case, the increases averaging 14 per cent for the dogs and 23 per cent for the ducks. In the experiments on the ducks, 1.1 per cent of the guanidine nucleus appears to have been methylated; in the experiments on dogs, 2.5 per cent methylation was obtained on those not receiving meat, and 14.6 per cent methylation on those receiving meat.
2. In 20 experiments upon 5 dogs, 7 ducks and 6 rabbits, it was found that the total creatinine excretion increased after the hypodermic or intravenous administration of arginine carbonate in all but one, or possibly two cases. The increases observed averaged 22.5 per cent for the dogs, and 5.2 per cent for the ducks; in rabbits, the hourly excretion of creatinine was more than doubled by the arginine injection (intravenous). These increases represent successful methylation of 4.5, 2.5, and 2.3 per cent of the guanidine nucleus injected. In control experiments, in which the solvent alone was injected, no consistent effect upon the creatinine output was noted.
3. The feeding of arginine was found to have no effect upon the excretion of preformed creatinine in dogs or birds (7 experiments). When administered parenterally, however, slight positive effects were noted in dogs in 6 experiments and in rabbits in 5 of 6 experiments. No effect was noted in 3 experiments upon birds.
4. Racemic arginine gave practically the same results as *d*-arginine. In two feeding experiments on dogs, there was an excess excretion of creatinine, equivalent to 6 per cent of the guanidine nucleus given, while in the same dogs after injection of arginine, the excess creatine excreted averaged 17.3 per cent * of the guanidine nucleus administered.
5. In 9 experiments upon rabbits it was found that the intravenous injection of arginine increased in all cases the creatine content of the muscle, as determined by analyzing the muscles of the right leg, before injection and the muscles of the left leg after injection. The increases corresponded to 8 to 25 per cent of the injected arginine, averaging 14.5 per cent. Control experiments indicated that neither the anesthetic used, the surgical operations, nor the solvent injected exerted any effect upon the creatine content of muscle.

There appears to be no valid reason for regarding Thompson's experiments other than a positive demonstration that the administration of arginine has brought about an increased formation of creatine.†

* In Hunter's review [19] the statement is made (p. 615) that "in Thompson's experiment the extra creatine never corresponds to more than a small fraction—from 1.1 to 4.5 per cent—of the guanidine nucleus introduced." This is plainly in error, since many larger percentages of methylation were reported in Thompson's work. It is a curious inconsistency that on page 599 of his review, Hunter is willing to accept a conversion of 5 per cent of ingested creatine into creatinine as proof of the possibility of such a conversion.

† The significance of the urinary creatine and creatinine excretions is enhanced by the fact that creatine formed in the body is not known to undergo any catabolic change except conversion into creatinine. They appear to represent, therefore, a measure of the rate of creatine formation in the body.

The most direct interpretation of them is that this increased formation of creatine was at the expense of the administered arginine, *i.e.,* the result of a chemical transformation. Hunter considers it just as reasonable that the creatine formed was the effect of a general amino acid stimulation, *i.e.,* the specific dynamic action. However, the specific dynamic action of amino acids has only been investigated as a calorigenic phenomenon, The assumption that it involves the endogenous catabolism rests upon no experimental evidence and is inherently improbable, since the characteristic end products of this catabolism are not affected in their rate of excretion by protein feeding, nor is creatine generally produced by protein feeding.

In substantiation of his amino-acid stimulation theory of creatine formation, Hunter refers to the work of Gross and Steenbock,[47] which confirms by feeding experiments upon pigs the work of Thompson on dogs, ducks, and rabbits. Gross and Steenbock observed distinct increases in the creatine excretion of pigs upon feeding arginine, but they obtained effects averaging 25 per cent greater from casein of equivalent arginine content. Hunter interprets this as indicating that the increase in creatine excretion obtained is a general amino acid stimulation rather than a result of the exogenous production of creatine from arginine. But surely the stimulating effect of 100 grams of casein would be much more than 25 per cent greater than the stimulating effect of 4.08 grams of arginine. The explanation of the greater creatine formation from casein than from equivalent amounts of arginine offered by Gross and Steenbock themselves, *i.e.,* that it is due to the phosphoric acid liberated from the casein in metabolism, seems adequate, since they have shown that even small amounts of acids induce creatinuria in pigs or increase a creatinuria already existent. This explanation receives substantial support from some experiments with cystine by the same investigators. The administration of 4.08 grams of cystine to a pig on a ration of starch invariably increased the excretion of creatine, but if sodium acetate (25 grams) was given simultaneously no such effect was observed. The sodium acetate furnished the body with sufficient base to neutralize the sulfuric acid produced from the oxidation of cystine. The effect of arginine and casein, on the other hand, was not inhibited by the simultaneous administration of sodium acetate. The contrast in the behavior of arginine and cystine on creatinuria favors the view that the arginine effect is specific and not due to a general amino acid stimulation.

McCollum and Steenbock [48] have noted differences among different

[47] Gross, E. G., and Steenbock, H., *J. Biol. Chem.,* 1921, xlvii, 33.
[48] McCollum, E. V., and Steenbock, H., *J. Biol. Chem.,* 1912, xiii, 209.

proteins with respect to the effect upon creatine production in the pig. In particular, they found that the protein of linseed oil meal was much more effective in this respect than the protein from corn. With no knowledge of the relative arginine content of these protein mixtures, they nevertheless believed that it was an arginine effect that they were observing. Later arginine analyses upon these feeds were reported by Hamilton, Uyei, Baker and Grindley,[49] from which it appears that 15.92 per cent of the nitrogen of linseed oil meal is in the form of arginine, while only 8.73 per cent of the nitrogen of corn is in this form.

In the later work of Steenbock and Gross,[47] casein was used in most of the experiments, though in one experiment 83.3 grams of an impure preparation of edestin was used with a very marked effect on the excretion of creatine, which rose from a level of 52 mgms. per day (expressed as creatinine) to 161 mgms. on the day of edestin feeding, 620 mgms. on the day following, and 74 mgms. on the third day. This effect was comparable with that secured with about 200 mgms. of casein. While casein contains 7.41 per cent of its total nitrogen in the form of arginine, edestin contains 27.05 per cent.[50] Gibson and Martin [37] failed to obtain any effect upon the creatine excretion of a boy of the substitution of edestin for 80 per cent of the protein in a basal diet, the composition of which is not given.

The effect of the feeding of an arginine-rich as compared with an arginine-poor protein upon the creatine content of muscle was investigated by Myers and Fine.[51] They analyzed the muscles of a number of rats, some of which had been fed upon rations containing casein as the sole protein, while some had been fed upon rations containing edestin as the sole protein. The results indicate a slightly higher average percentage of creatine in the muscles of the edestin rats, but the authors were not inclined to attach any significance to it. The experiment is generally referred to as a negative one,[19] though a statistical analysis of the results indicates that it must reasonably be considered as significantly positive. If the individual results are arranged in the order of decreasing contents of creatine, the result is as in Table 25.

Of the 9 highest results, only 2 were obtained with casein rats, while the lowest 10 results were all from casein rats. This can hardly be considered a random distribution, such as would have been obtained if the main factors involved were the individuality of the rats or the analytical errors of the method.

[49] Hamilton, T. S., Uyei, N., Baker, J. B., and Grindley, H. S., *J. Amer. Chem. Soc.*, 1923, xlv, 815.
[50] Van Slyke, D. D., *J. Biol. Chem.*, 1911, x, 15; 1913, xvi, 531.
[51] Myers, V. C., and Fine, M. S., *J. Biol. Chem.*, 1915, xxi, 399.

TABLE 25. *Results of Myers and Fine on the Creatine Content of the Muscle of Rats Fed Proteins High and Low in Arginine.*

Order	Creatine Content	Protein Fed	Order	Creatine Content	Protein Fed
1	0.476	edestin	10	0.464	casein
2	0.476	"	11	0.464	"
3	0.470	casein	12	0.458	"
4	0.470	edestin	13	0.454	"
5	0.470	"	14	0.453	"
6	0.468	"	15	0.453	"
7	0.467	"	16	0.453	"
8	0.465	casein	17	0.453	"
9	0.464	edestin	18	0.453	"
			19	0.453	"

The eleven casein rats had an average content of 0.4582 ± 0.0012 per cent creatine in the muscle. The seven edestin rats had an average percentage of 0.4701 ± 0.0011 and were in general a much more uniform group. The difference in percentage with its probable error was 0.0119 ± 0.0016. A difference exceeding its probable error by more than seven times may be assumed to possess a high degree of significance according to ordinary statistical standards, equivalent to odds greater than 420,000 to 1. The average moisture content of the muscle of the casein rats was 73.54 per cent, and of the arginine rats, 74.24, so that the results of the creatine analysis of the muscle, if calculated to tissue of uniform water content, would show even a greater difference in favor of the edestin rats. The conclusion appears inevitable that the analytical data of Myers and Fine, contrary to current belief, establishes a degree of probability amounting to practical certainty that the difference in protein feeding between the two groups of rats has occasioned a difference in the creatine content of the muscle. This may reasonably be interpreted as indicating a probable exogenous origin of creatine, and in particular it may be considered as legitimate evidence that arginine is a precursor.

In their work upon the synthesis of ornithine in the body of the fowl, Crowdle and Sherwin [52] observed a marked reduction in the excretion of total creatinine upon giving benzoic acid alone, and a marked increase in the excretion of total urinary nitrogen, simultaneous with the appearance of ornithuric acid in the urine. Although the experiment is interpreted by Crowdle and Sherwin as a demonstration of a synthesis of ornithine and its conjugation with a part of the benzoic acid, the picture presented by the analytical data is not inconsistent with the view that the endogenous catabolism was increased by benzoic acid feeding, that the arginine thus produced, *as well as the arginine normally*

[52] Crowdle, J. H., and Sherwin, C. P., *J. Biol. Chem.*, 1923, lv, 365.

serving as a precursor of muscle creatine, was split by kidney arginase into ornithine, and the latter used for detoxication purposes. The experiment, therefore, does not stand in contradiction to the earlier work of Thomas,[53] who was not able to demonstrate a synthesis of ornithine by the hen, while it furnishes an interesting confirmation of the view that arginine is the precursor of creatine. Since the data of Crowdle and Sherwin have not hitherto been cited in this connection they have been summarized in Table 26.

TABLE 26. *The Effect of Benzoic Acid Upon the Excretion of Total Creatinine by the Chicken. From Crowdle and Sherwin.*

Day	Benzoic Acid Fed	Total Urinary Nitrogen	Creatinine and Creatine Nitrogen	Combined Benzoic Acid Excreted †
	gms.	gms.	mgms.	gms.
2	0	0.764	29	...
3	0	0.622	29	...
4	0	0.642	33	...
5	1	0.952	31	0.37
6	1	0.897	29	0.51
7	1	0.584	18	0.41
8	0	0.405	9	0.23
9	0	0.366	7	...
10	1	0.521	15	0.47
11	1	0.501	12	0.43
12	1	0.609	13	0.17
13	1	0.581	10	0.12
14	0	0.574	9	...
15	0	0.541	15	...
16	0	0.749*	17	...
17	0	0.553	19	...
18	0	0.529	22	...

* 1 gm. of histidine was given on this day.
† Identified, at least in part, as ornithuric acid.

The conclusion that arginine is a precursor of creatine is in agreement with the results of an investigation by Henderson on the effect of parathyroidectomy on the muscles of dogs.[54] She found that after the removal of the parathyroids, there is a fall in the total and the free guanidine of muscle, and a rise in the content of creatine, both absolutely and in relation to the total nitrogen. Of more direct significance are the results of Jansen [55] to the effect that increased tonus in frog muscle is accompanied by a disappearance of arginine and the production of a corresponding amount of creatine.

Such a reciprocal relation between arginine and creatine is implied from analyses of the extractives of the tissues of vertebrates as com-

[53] Thomas K., *Centr. Physiol.*, 1914, xxviii, 769.
[54] Henderson, P. S., *J. Physiol.*, 1918, lii, 1.
[55] Jansen, B. C. P., *Arch. nederland Physiol.*, i, 618; *J. Chem. Soc.*, 1917, cxii, I, 606; *Chem. Abst.*, 1918, xii, 932.

pared with invertebrates. In a series of articles by Kutscher and Ackermann [56] it has been shown that the tissues of molluscs, crustaceans, and insects contain arginine (or putrescin, a derivative of arginine), but no creatine, in marked contrast to the tissues of vertebrates which contain creatine, but no free arginine. In this respect, invertebrate tissue resembles plant tissue. The results of Okuda [57] also bear out this distinction between vertebrates and invertebrates. He concludes that, of the four extractives, creatine, histidine, arginine, and betaine, the first two are characteristic of vertebrate tissue and the last two of invertebrate tissue.

In conclusion, it appears that the ability of vertebrate tissues, in particular muscle tissues, to convert arginine into creatine has been amply demonstrated, not only in experiments upon arginine itself, but also in experiments upon glycocyamine, a probable metabolite of arginine. The work of Thompson is particularly convincing; if it had been concerned with a problem about which so much controversy had not been stirred up by the publication of many small scale experiments yielding inconclusive results, it would in all probability be considered a monumental contribution that settled beyond reasonable doubt the problem with which it was concerned. The confirmatory data of Steenbock and Gross, of Myers and Fine, and of Crowdle and Sherwin completes the imposing array of evidence that arginine is the precursor of creatine in animal metabolism, while much interesting circumstantial evidence, such as that of Henderson, Jansen, Kutscher and Ackermann, is explainable on this basis. The final conclusion of Hunter on this question, after a full consideration of the evidence up to 1922, is incomprehensible. Hunter says: "In the face of such *almost uniformly negative results* one is almost forced to assume that, *if creatine is related to arginine at all,* its mother substance is not free arginine, but the still combined arginine of the muscle protein." The italics are ours.

The Relation of the Parathyroid Glands to Arginine Metabolism

There is considerable indirect and inferential evidence, and some evidence of a more direct bearing, that the parathyroid glands are related to the metabolism of arginine. This evidence concerns the relation of the parathyroid glands, first, to the metabolism of guanidine and methylated guanidine, and second, to creatine metabolism. In all probability arginine is the most important, if not the only, dietary precursor of

[56] Kutscher, F., *Z. Biol.,* 1914, lxiv, 240; Ackermann, D., *ibid.,* 1920, lxxi, 193; 1921, lxxiii, 319; 1922, lxxiv, 67.
[57] Okuda, Y., *J. Coll. Agr. Imper. Univ. Tokyo,* 1919, vii, 55.

creatine, while it appears to be the only considerable source of guanidine in food materials.[58]

For reviews of the many scientific contributions to the question of the functions of the parathyroid glands, the reader is referred to Boothby [59] and to Simpson,[60] although many important papers have appeared since the publication of these contributions. One of the first theories that was proposed was that the parathyroid glands are concerned in calcium metabolism and utilization, and that the tetany produced by extirpation of these glands is closely dependent upon a disturbance of the calcium content of the blood and tissues. While the evidence leading to the inception of this theory was contributed by various investigators, it has undoubtedly received its greatest support and its most definite formulation by W. G. MacCallum and his associates. The theory rests upon demonstrations of a marked lowering of the calcium content of the blood after parathyroidectomy, of the incidence of tetany when the blood calcium decreases to a certain more or less definite level, of the low calcium content of the brain and nervous system in cases of fatal tetany, of the favorable therapeutic and prophylactic effects of the administration of calcium salts, and of the sedative effect of the calcium ion upon the nervous system. MacCallum and Voegtlin [61] in 1909 pictured the relation of calcium salts to the parathyroid glands as follows: "These salts have a moderating influence upon the nerve cells. The parathyroid secretion in some way controls the calcium exchange in the body. It may possibly be that, in the absence of the parathyroid secretion, substances arise which can combine with calcium, abstract it from the tissues, and cause its excretion, and that the parathyroid secretion prevents the appearance of such bodies. The mechanism of the parathyroid action is not determined, but the result [of its removal], the impoverishment of the tissue with respect to calcium, and the consequent development of hyperexcitability of the nerve cell, is proven. Only the restoration of calcium to the tissues can prevent this."

Since its definite formulation, the calcium deficiency theory has languished due to the accumulation of many facts apparently inconsistent with it, and in text and reference books dating no later than 1922 to 1924 it is not ordinarily endorsed, though obviously it embodies at least a partial explanation of parathyroid function. However, in the last few years (1924 to 1926) that have seen the successful isolation of active fractions from parathyroid tissue, the calcium deficiency theory

[58] Barger, G., "The Simpler Natural Bases," London and New York, 1914, pp. 78-80.
[59] Boothby, W. M., *Endocrinology*, 1921, v, 403.
[60] Simpson, S., "Endocrinology and Metabolism," New York and London, 1922, vol. I, 509.
[61] MacCallum, W. G., and Voegtlin, C., *J. Exper. Med.*, 1909, xi, 118.

has evidently received an enormous impetus. Hanson,[62] Berman,[63] Collip [64] and Fisher and Larson [65] have recently prepared extracts of parathyroid glands which have proven to be completely curative of tetania parathyreopriva and to possess the power of raising the concentration of calcium in the blood to normal. Collip in particular has presented a mass of evidence on the effectiveness of his preparations. Impressed with the close parallelism between the concentration of calcium in the blood serum of parathyroidectomized dogs and the clinical symptoms exhibited, with and without treatment with his hormone preparation, Collip concludes:

"The normal function of the parathyroid glands would appear to be related to the regulation of certain phases of mineral metabolism through a direct control of the calcium level of the blood. While it may later develop that these glands have other functions, there would seem to be no need at present to ascribe any function to them other than that of regulators of calcium metabolism. Loss of this function following removal of the glands is the sole cause of tetania parathyreopriva, in the opinion of the writer." Not the least interesting of the experimental findings of this investigator is the demonstration that the administration of parathyroid hormone to normal dogs induces a hypercalcemia proportionate in degree to the amount of hormone given.

It is beyond the province of this discussion to consider all of the evidence relating tetanic symptoms to blood calcium, or to consider the many confirmatory experiments of the therapeutic effect of calcium salts and of the corrective effect of the administration of parathyroid hormone upon calcium metabolism. Admittedly it makes an imposing argument for the theory propounded. However, the theory does not appear to explain many of the observed facts relating to the physiological conditions induced by parathyroidectomy, and it fails to consider other metabolic disturbances than those relating to calcium. For example, Greenwald observed a marked diminution in the excretion of phosphorus through the kidneys after parathyroidectomy [66] and an increase in the phosphorus content of the blood,[67] while Underhill and Blatherwick [68] showed that in parathyroidectomized dogs killed in tetany the liver is

[62] Hanson, A. M., *Military Surgeon*, 1923, lii, 280; 1924, liv, 76, 218, 554; lv, 719; *Proc. Soc. Exp. Biol. and Med.*, 1925, xxii, 560.

[63] Berman, L., *Proc. Soc. Exp. Biol. and Med.*, 1924, xxi, 465; *Amer. J. Physiol.*, 1925-26, lxxv, 358.

[64] Collip, J. B., *J. Biol. Chem.*, 1925, lxiii, 395; Collip, J. B., Clark, E. P., and Scott, J. W., *J. Biol. Chem.*, 1925, lxiii, 439; Collip and Clark, *ibid.*, 1925, lxiv, 485.

[65] Fisher, N. F., and Larson, E., *Proc. Soc. Exp. Biol. and Med.*, 1925, xxii, 447; *Amer. J. Physiol.*, 1925-26, lxxv, 93.

[66] Greenwald, I., *J. Biol. Chem.*, 1913, xiv, 363.

[67] Greenwald, I., *J. Biol. Chem.*, 1913, xiv, 369.

[68] Underhill, F. P., and Blatherwick, N. R., *J. Biol. Chem.*, 1914, xviii, 87. *See also* Underhill and Nellans, C. T., *ibid.*, 1921, xlviii, 557.

glycogen-free, while the blood sugar content is markedly lowered or even reduced to zero.

Parathyroidectomized dogs may be preserved by therapeutic methods bearing no apparent relation to calcium metabolism. Luckhardt and Rosenbloom [69] observed that dogs in parathyroid tetany could repeatedly be relieved by inducing a vigorous diuresis through the intravenous injection of large quantities of calcium-free Ringer's solution, while Dragstedt and Sudan [70] obtained analogous results by the use of a modified Ringer's solution in which strontium chloride replaced calcium chloride. In fact, the oral administration of strontium lactate, while not so effective as the administration of the calcium salt, was capable of curing parathyroid tetany and preserving the life of parathyroidectomized dogs for at least 24 days. These results were in substantial agreement with those reported earlier by Swingle and Wenner,[71] who were able to keep their operated dogs alive indefinitely by strontium administration *per os*. These investigators explain the favorable effects of strontium as being due to a decreased permeability of the gut to calcium excretion, though no evidence is presented of this nature. The explanation was suggested by a paper by Salvesen [72] in which it is claimed that "in parathyroidectomized dogs there is a lowered threshold for the excretion of calcium in the intestines, and this is apparently the cause of the calcium deficiency and thereby of all the symptoms." However, a diligent search of the paper fails to reveal any evidence of an abnormal excretion rate for calcium in parathyroidectomized dogs.*

A significant experiment was reported by Jones,[73] who was able to prevent tetany and greatly prolong the life of parathyroidectomized dogs by the daily administration of 20 cc. of cod liver oil before the operation. Although no tetany supervened, the concentration of calcium in the blood of the treated animals fell as rapidly as in untreated animals; in fact, the lowest levels were attained in the blood of the treated dogs that lived the longest. The data indicate that some factor in addition to, or other than, the low concentration of serum calcium is responsible for parathyroid tetany. Binger [74] has also noted greatly diminished blood

[69] Luckhardt, A. B., and Rosenbloom, P. J., *Proc. Soc. Exper. Biol. and Med.*, 1921-22, xix, 129.

[70] Dragstedt., L. R., and Sudan, A. C., *Amer. J. Physiol.*, 1926, lxxvii, 307.

[71] Swingle, W. W., and Wenner, W. F., *Amer. J. Physiol.*, 1926, lxxv, 378.

[72] Salvesen, H. A., *J. Biol. Chem.*, 1923, lvi, 443.

* The return of the calcium level of the blood to the pre-level, following the injection of calcium salts, appears no more rapid in the parathyroidectomized dogs than in the normal dogs later investigated by Salvesen and associates (Salvesen, Hastings, and McIntosh, *J. Biol. Chem.*, 1924, lx, 327), in which the injected calcium left the blood in 3 to 6 hours. The assumption that there is a threshold level for the excretion of calcium by the intestine, and that a lowering of the threshold will lower the calcium level in the blood, is without experimental justification and, in fact, without analogy in kidney functioning. For example, the threshold value for sugar excretion bears no relation to the normal level of sugar in the blood.

[73] Jones, J. H., *J. Biol. Chem.*, 1926. lxx, 647.

[74] Binger, C. *J. Pharm. and Exper. Therap.*, 1917, xi, 105.

calcium unaccompanied by tetany in normal dogs, while Sloan [75] has noted high blood calcium simultaneous with tetany. These, and other instances of similar import that may be cited, indicate that blood calcium levels do not always parallel the condition of parathyroidectomized animals.

Dragstedt and his associates have accumulated considerable evidence that a proper control of intestinal conditions in parathyroidectomized dogs is all that is required in preventing tetany and preserving life. Dietary control aimed particularly at the maintenance of an aciduric type of intestinal flora was found to be particularly effective.[76] Lactose was much more effective than milk itself, while a similar effectiveness was demonstrated for galactose [77]; glucose, sucrose, and dextrin proved relatively ineffective. While the effectiveness of lactose in preventing tetany may be explained by its facilitating the absorption of calcium, on the basis, for instance, of Bergeim's recent findings,[78] Dragstedt and Sudan [79] emphasize the fact that it requires very large doses of calcium lactate orally (from 20 to 50 grams daily in a 10 kg. dog) to prevent tetany, and question seriously the inference that lactose could bring about any considerable increase in calcium absorption from the calcium-poor basal diets with which it had been used with success (for example, bread and water, or bread and 300 cc. of milk daily).

On the basis of such results, Dragstedt believes [76] that parathyroid tetany or depression is due to an intoxication, that the responsible toxic substance or substances comes chiefly from the gastro-intestinal tract and is probably the result of bacterial proteolysis, that the function of the parathyroid glands is to prevent intoxication by these poisons, but that the parathyroid hormone is not necessary for life if other means are taken to prevent intestinal toxemia. The administration of kaolin [79] was found to be effective in controlling parathyroid tetany, probably, according to the theory, because of its capacity of adsorbing toxic products of bacterial growth. In further agreement with the theory was the finding [80] that small doses of yellow phosphorus, a specific liver poison, would induce tetany in parathyroidectomized dogs, apparently by interfering with the detoxicating function of the liver; also, the intravenous injection of small amounts of material from an obstructed intestinal loop into operated dogs induced peripheral nerve responses not obtained from normal dogs. Of much the same significance are the experiments of

[75] Sloan, H., *Amer. J. Physiol.*, 1926, lxxix, 100.
[76] Dragstedt, L. R., and Peacock, S. C., *Amer. J. Physiol.*, 1923, lxiv, 424.
[77] Inouye, T., *Amer. J. Physiol.*, 1924, lxx, 524.
[78] Bergeim, O., *J. Biol. Chem.*, 1926, lxx, 35.
[79] Dragstedt, L. R., and Sudan, A. C., *Amer. J. Physiol.*, 1926, lxxvii, 314.
[80] Dragstedt, L. R., Phillips, K., and Sudan, A. C., *Amer. J. Physiol.*, 1923, lxv, 368.

Oldberg and Ivy [81] in which colectomy was found to delay greatly the onset, and to moderate considerably the symptoms, of tetany induced by the removal of the thyroid-parathyroid gland substance. However, the rôle of the liver as a detoxifying agent in tetany seems to be questioned by the results of Blumenstock and Ickstadt,[82] who found that, in parathyroidectomized dogs with an Eck fistula, tetany was more readily controlled by calcium administration than in similar dogs without any disturbance of the liver circulation, although the Eck fistula dogs did not pass into a condition in which calcium treatment could be suspended, as otherwise would have occurred.

In view of the experimental work just described, yielding results not explainable, on the basis of available information, by the calcium deficiency theory, and pointing to an intoxication as the probable cause of the symptoms of tetania parathyreopriva, it seems a fair judgment that the calcium deficiency theory must still be considered as being on trial until it can be shown that the undoubted disturbances in calcium metabolism are the *cause* of the tetany, not merely *one of its effects.*

Although the advocates of the intoxication theory in this country are not prepared to announce what particular toxins may be responsible for the symptoms produced after extirpation of the parathyroid glands, there are indications, if nothing more, that the responsible toxins may be guanidine or guanidine derivatives.

In 1912 and 1913, W. F. Koch [83] reported the isolation and identification of methyl guanidine and other guanidine bases in the urine of parathyroidectomized dogs, and in 1916 Burns and Sharpe [84] obtained much larger amounts of guanidine and methyl guanidine from the blood and urine of parathyroidectomized dogs than from the blood and urine of normal dogs. In 1920 Findlay and Sharpe [85] isolated dimethylguanidine from the urine of a woman suffering from idiopathic tetany. In the same year Sharpe [86] observed a greatly increased excretion of dimethylguanidine in the feces in four cases of idiopathic tetany as compared with six normal cases, while Nattrass and Sharpe [87] in the following year reported the presence of considerable amounts of dimethylguanidine in the urine in two cases of adult tetany.

However, the methods used by these investigators for the isolation and identification of guanidines have been severely criticized by Green-

[81] Oldberg, E., and Ivy, A. C., *Amer. J. Physiol.*, 1926, lxxvi, 226.
[82] Blumenstock, J., and Ickstadt, A., *J. Biol. Chem.*, 1924, lxi, 91.
[83] Koch, W. F., *J. Biol. Chem.*, 1912, xii, 313; 1913, xv, 43.
[84] Burns, D., and Sharpe, J. S., *Quart. J. Exp. Physiol.*, 1916, x, 345.
[85] Findlay, L., and Sharpe, J. S., *Quart. J. Med.*, 1919-1920, xiii, 431.
[86] Sharpe, J. S., *Biochem. J.*, 1920, xiv, 46.
[87] Nattrass, F. J., and Sharpe, J. S., *Brit. Med. J.*, 1921, II, 238.

wald,[88] who pointed out that the method of Engeland [89] for the isolation of methylguanidine, involving precipitation with mercuric salts, will bring about an oxidation of any creatine present to methyl-guanidino-glyoxylic acid, which on evaporation of its solutions, decomposes into methyl guanidine and oxalic acid.[90] Hence, "it is quite evident that the isolation of methylguanidine from materials that may contain creatine must not be attempted by methods that include precipitation with mercuric acetate or mercuric chloride and sodium acetate." This criticism applies to Koch's work and to the experiments of Burns and Sharpe. Against the later investigations, in which the use of mercuric salts was avoided, Greenwald shows that the identification of the isolated crystals as picrates or gold salts of guanidines was not sufficiently definite to warrant any definite conclusion as to their exact composition. He himself was unable to detect methylguanidine in the urine of parathyroidectomized dogs, though he admits that his analytical method was very imperfect; and in a later investigation [91] he could obtain no indication, in biological experiments, of the existence of a toxin in the blood of such dogs.

Greenwald's criticisms cast considerable doubt upon all previously reported isolations of guanidine bases from the blood and excretions of tetany cases, either experimental or clinical. Evidently, none of the methods of separation used are satisfactory, and the demonstration of an increased guanidine excretion in tetany, if such there is, must wait upon the formulation of more accurate chemical methods. It is to be hoped that the colorimetric method of Marston,[92] but recently reported, will prove to be adequate for this purpose. In preliminary experiments with it, Marston has been unable to detect free guanidine bases in muscle, but has observed their presence in traces in normal urine. Frank and Kühnau [93] have recently reported the finding of methyl- and dimethylguanidine in the urine from two cases of parathyroid tetany, while the same method of isolation (as picrate or carbonate) failed to reveal the presence of such bases in normal urine.*

Aside from these chemical investigations, there is considerable in-

[88] Greenwald, I., *J. Biol. Chem.*, 1924, lix, 329. *See also* White, F. D., *J. Biol. Chem.*, 1927, lxxi, 419.

[89] Engeland, R., *Z. physiol. Chem.*, 1908, lvii, 49; *Z. Untersuch. Nahrungs-u. Genussmittel*, 1908, xvi, 658.

[90] Dessaignes, M., *Compt. rend. Acad.*, 1854, xxxviii, 839; Baumann, L. and Ingvaldsen, T., *J. Biol. Chem.*, 1918, xxxv, 277; Greenwald, I., *J. Amer. Chem. Soc.*, 1919, xli, 1109.

[91] Greenwald, I., *J. Biol. Chem.*, 1924, lxi, 33.

[92] Marston, H. R., *Austrlian J. Exptl. Biol. Med. Sci.*, 1924, i, 99; 1925, ii, 57, 93.

[92] Marston, H. R., *Australian J. Expl. Biol. Med. Sci.*, 1924, i, 99; 1925 ii, 57, 93.

* In a later paper, using another analytical method in which phosphotungstic acid is the final precipitant, Kühnau (*Arch. exp. Path. Pharm.*, 1926, cxv, 75) reports considerable increases in methylated guanidines in the blood and urine of parathyroidectomized dogs. However, Noether (*ibid.*, 1926, cxi, 38), using cats as experimental subjects and Greenwald's analytical methods, was unable to obtain significant differences in the urinary excretion of guanidine bases between normal and thyroparathyroidectomized animals.

ferential evidence relating parathyroid tetany to guanidine intoxication. As the result of an extensive comparison of the symptomatology of guanidine and methylguanidine poisoning, idiopathic tetany and post-operative tetany in man, and tetania parathyreopriva, Paton and Findlay and their associates [94] concluded that all of these conditions possess an identical etiology, and that the last three forms of tetany are due to the development of guanidine bases in the body as the result of some interference with the function of the parathyroid glands in controlling guanidine metabolism and, by this means, the tonus of the skeletal muscles. These investigators point out that no drug, other than guanidine and methylguanidine, is known that can effect a decided increase in the excitability of the motor nerve endings to the constant electric current. It has also been noted that the ages at which creatine, a guanidine derivative, is normally present in the urine of children are the ages at which the incidence of tetany is greatest. Confirmation of the similarity in symptomatology between guanidine intoxication and parathyroid tetany may be found in papers by Burns and Watson,[95] Klinger,[96] and Frank, Stern, and Nothmann,[97] while Frank and Stern [98] have shown that methylguanidine acts qualitatively exactly like guanidine with reference to motor irritability. On the other hand, Dragstedt, Phillips, and Sudan [80] were not able to produce the typical symptoms of tetany in normal dogs by the injection of guanidine or methylguanidine in the doses used. However, with parathyroidectomized animals, guanidine and methylguanidine produced symptoms of tetany in doses that had little or no effect on unoperated dogs. Buchanan and Garven [99] have noted certain differences between parathyroid tetany and guanidine tetany with reference to the electrical response of the peripheral nerves. Bayer and Form [100] also doubt the identity of the two conditions, while Collip [101] observed no similarity in the variations of non-protein nitrogen and urea in the blood of parathyroidectomized dogs and of dogs poisoned with guanidine.

However, it must be admitted that the symptoms of experimental guanidine poisoning may differ from the symptoms of a guanidine intoxication induced naturally. The divergent results reported by different investigators following guanidine administration indicate that the mode

[94] Paton, D. N., Findlay, L., and Burns, D. *J. Physiol.*, 1914-15, xlix, p. xvii; Paton, and Findlay, *Quart. J. Exper. Physiol.*, 1916, x, 203 315, 377; Paton, Findlay and Watson, A., *ibid.*, 1916, x, 233, 243; Wishart, G. M., *ibid.*, 1916, x, 355; Burns, D., *ibid.*, 1916, x, 361. *See also* ([84]) and Paton, *Rpt. Brit. Assoc. Adv. Sci.*, 1919 294.
[95] Burns, D., and Watson, A. M., *J. Physiol.*, 1918-19, lii, 88.
[96] Klinger, R., *Arch. exptl. Path. Pharm.*, 1921, xc, 129.
[97] Frank, E., Stern, R., and Nothmann, M., *Z. exptl. Med.*, 1921, xxiv, 70.
[98] Frank, E., and Stern, R., *Arch. exptl. Path. Pharm.*, 1921, xc, 149.
[99] Buchanan, D. N., and Garven, H. S. D., *J. Physiol.*, 1926, lxii, 115.
[100] Bayer, G., and Form, O., *Z. ges. exptl. Med.*, 1924, xl, 445.
[101] Collip, J. B., *J. Biol. Chem.*, 1926, lxvii, 679.

of administration and the dosage must be factors of importance in determining the type of reaction obtained, and, therefore, that the symptoms of a natural intoxication cannot always be duplicated experimentally. Hence, it would appear that more significance should be attached to the similarities noted between the symptoms of experimental guanidine poisoning and the symptoms of parathyroidectomy than should be accorded the reported dissimilarities.

An interesting and significant series of investigations of the metabolic changes induced by the administration of guanidine bases to rabbits has been reported by Watanabe, working in Underhill's laboratory. A typical tetany was readily induced by the administration of guanidine hydrochloride, similar in its clinical symptoms to that of tetania parathyreopriva.[102] At the same time, a hypoglycemia was demonstrable in most cases, recalling the similar symptom that Underhill and Blatherwick observed following parathyroidectomy.* However, in the latter case the hypoglycemia was much more marked. The excretion of ammonia in the urine increased markedly, while the hydrogen-ion concentration of the urine decreased after administration of the drug,[103] simulating the urinary conditions observed by many, but not all, investigators of the derangements of metabolism following surgical removal of the parathyroid glands.

Of particular significance are the experimental observations of the relations between guanidine intoxication and calcium metabolism. Watanabe observed[104] after guanidine poisoning in rabbits marked increases in the phosphate of the blood and frequent though inconstant decreases in the calcium. György and Vollmer[105] and Gollwitzer-Meier[106] have also reported decreases in serum calcium concentration following guanidine intoxication, while Behrendt[107] was not able to confirm this observation. In all probability the size of the dosage of guanidine is a determining factor in the effect on the calcium content

[102] Watanabe, C. K., *J. Biol. Chem.*, 1918, xxxiii, 253.

* Attention may be directed here to several interesting relations that have been observed between arginine, guanidine, and insulin, the pancreatic hormone. The lowering of blood sugar by guanidine administration is an insulin-like action, which is also displayed by certain guanidine derivatives, according to Frank, Nothmann and Wagner (*Klin. Woch.*, 1926, v. 2100). Agmatine, the amine corresponding to arginine, lowers blood sugar and is less toxic than guanidine. They were able to synthesize an arginine or guanidine derivative (synthalin) so active in this respect that a few milligrams *per os* caused hypoglycemic convulsions. In a previous article Frank (*ibid.*, 1924, iii, 955) suggested that the active grouping in the insulin molecule might be the guanidine group, while Collip (*J. Biol. Chem.*, 1923, lviii, 163) has also suggested, on the basis of his own experimental work, the possibility "that insulin, guanidine, and certain constituents of plant extracts are related chemically in some fundamental manner." A more definite indication of such a relationship is afforded by the preliminary report of experiments by Sandberg and Brand (*Proc. Soc. Exp. Biol. Med.*, 1927, xxiv, 373), showing a high proportion (12 per cent) of arginine in insulin, and demonstrating that the alkali in activation of insulin is associated with the splitting off of urea.

[103] Watanabe, C. K., *J. Biol. Chem.*, 1918, xxxiv, 51, 65.

[104] Watanabe, C. K., *J. Biol. Chem.*, 1918, xxxiv, 73; xxxvi, 531.

[105] György, P., and Vollmer, H., *Arch. exptl. Path. Pharm.*, 1922, xcv, 200.

[106] Gollwitzer-Meier, K., *Z. ges. exptl. Med.*, 1924, xl, 50.

[107] Behrendt, H., *Klin. Wochenschr.*, 1925, iv, 1600.

of the blood. It was observed by Dragstedt and Sudan [108] that in thyroparathyroidectomized dogs which have been kept free from tetany over a period of several weeks by dietary control, there is no marked decrease in the content of calcium of the blood serum following immediate or prolonged intoxication produced by guanidine hydrochloride. However, if parathyroid tetany and guanidine tetany are identical conditions, it would be expected that the dietary factors controlling the former would also control the latter.

Klinger [96] was not able to relieve the symptoms of guanidine poisoning by the administration of calcium salts, and Collip and Clark [109] could not demonstrate any antagonistic action between guanidine and the parathyroid hormone obtained by his method. The injection of the latter into normal dogs together with guanidine appeared to be as effective in the production of hypercalcemia as the injection of the hormone alone. On the other hand, Burns and Watson [95] found that calcium salts removed the interference with vago-cardiac inhibition produced either by guanidine intoxication or parathyroid tetany, and Fühner [110] observed that calcium salts suppress the increase in tonus and the fibrillar twitching of skeletal muscle brought about by guanidine poisoning. Also, Palladin and Griliches [111] were able to prevent the appearance of tetany after guanidine injection by the simultaneous administration of calcium salts.

It seems clear that a relation of the parathyroid glands to guanidine metabolism has not been demonstrated. However, there are many circumstantial indications that such a relation exists. It is unfortunate that the reported experimental observations on the effects of guanidine poisoning are so contradictory, a situation that must be due to incidental factors, such as the dosage and the condition of the experimental subjects, that need further investigation. In determining whether parathyroid tetany is due to guanidine poisoning, it seems evident that the effects of repeated small doses of guanidine would be of more significance than the effects of massive doses.

The relation of the parathyroids and of the guanidine bases to creatine metabolism represents another problem pertinent to a discussion of the metabolism of arginine. Greenwald [112] has shown that parathyroidectomy occasions an excretion of creatine in the urine, a finding confirmed by Burns,[94] while Woodman [113] observed an increased

[108] Dragstedt, L. R., and Sudan, A. C., *Amer. J. Physiol.*, 1926, lxxvii, 321.
[109] Collip, J. B., and Clarke, E. P., *J. Biol. Chem.*, 1925, lxiv, 485.
[110] Fühner, H., *Arch. exptl. Path. Pharm.*, 1925, cv, 265.
[111] Palladin, A., and Griliches, L. *Biochem. Z.*, 1924, cxlvi, 458.
[112] Greenwald, I., *Amer. J. Physiol.*, 1911, xxviii, 103.
[113] Woodman, Dorothy, *Biochem. J.*, 1925, xix, 595.

excretion of creatine in three out of four rats following parathyroid feeding. Reference has already been made to the very convincing results of Henderson [52] to the effect that in tetania parathyreopriva the creatine content of muscle is increased markedly. The simultaneous decrease in total and free guanidine bases is interesting, and the analytical values are remarkably consistent, but the significance of these findings is somewhat impaired by the unsatisfactory methods available for such an analysis.

Just as removal of the parathyroids increases the creatine content of muscle, so does guanidine injection also, according to a number of experiments upon dogs, cats, and hens reported by Wishart.[114] Palladin and Griliches [111] have obtained some very significant experimental results along this line. They found that guanidine tetany, as well as parathyroid tetany, caused the appearance of creatine in the urine of adult rabbits, or increased the creatinuria of puppies. The results with guanidine confirm some earlier results reported by Thompson.[115] It was further shown that the creatine content of the muscle of rabbits killed in guanidine tetany was distinctly increased over the normal, and that tetania parathyreopriva occasioned an increase in the content of creatine in the muscle of dogs. The tetany of rabbits due to guanidine injection could be suppressed by the simultaneous injection of calcium salts, and in such cases the creatine content of the muscle was not increased nor was the elimination of creatine in the urine disturbed. The increased excretion of creatine in guanidine tetany and in parathyroid tetany may be related to the increased creatine content of the muscles.

Hammett [116] demonstrated that the addition of parathyroid tissue to extracts of muscle tissue of albino rats retards the increase of creatinine formation normally taking place during incubation. This occurred in acid, neutral, or alkaline mixtures, while thyroid tissue, under similar conditions, was without effect. "Since the maximum retardation effect of the parathyroids occurs in solutions buffered to neutrality, while the maximum creatinine formation takes place at the same reaction, the conclusion is justified that this parathyroid effect is an expression of a direct influence of the parathyroids on creatine metabolism." This effect of the parathyroids is not readily correlated with the experimental results just considered, though they do strengthen the conclusion that these glands are intimately involved in creatine, and hence arginine, metabolism.

[114] Wishart, G. M., *J. Physiol.*, 1920, liii, 440.
[115] Thompson, W. H., *J. Physiol.*, 1917, li, 347.
[116] Hammett, F. S., *J. Biol. Chem.*, 1921, xlvii, 143.

HISTIDINE

The Catabolism of Histidine

Although histidine contains a distinctive heterocyclic ring, little definite information has been reported relating to its metabolic transformations in the body, probably because the imidazole ring is readily destroyed by the tissues, and because, until recently, the identification of imidazole compounds was difficult to obtain satisfactorily. In 1908, Engeland [117] obtained small amounts of histidine and imidazole aminoacetic acid * from human urine, though by far the largest fraction of the imidazole derivatives could not be identified. Heffer [118] also found small amounts of histidine in normal urine. More recently, Fürth [119] has reported unsuccessful attempts to identify a single imidazole compound from human urine. In 1922, Hunter [120] isolated 0.1 gram of histidine from a liter of urine from a case of measles, while Reinwein [121] reports its occurrence in the urine in advanced pulmonary tuberculosis and in pernicious anemia.

In 1874, Jaffé [122] isolated from the urine of a dog, which had been used for a long time in feeding experiments with nitrotoluol, an imidazole compound that he called urocanic acid (Urocaninsäure). Another occurrence of the same compound in the urine of a dog receiving experimental doses of sodium telluride was reported by Siegfried [123] in 1898, while Swain [124] in 1905 described a compound obtained from coyote urine apparently closely related to, if not identical with, urocanic acid. Apparently the dogs of Jaffé and Siegfried presented a rare anomaly of metabolism, since other dogs examined by Jaffé failed to excrete the acid; Hunter [125] also was unable to find a dog from which urocanic acid could be secured, and Hunter and Givens [126] were unable to prepare it from the urine of a coyote. Siegfried failed to find it in human urine. Hunter has shown that urocanic acid is β-4-imidazole acrylic acid, the α-β-unsaturated acid corresponding with the amino acid histidine. This acid had previously been synthesized by Barger and Ewins.[127]

The two recorded occurrences of urocanic acid in urine do not

[117] Engeland, R., *Z. physiol. Chem.*, 1908, lvii, 49.

* However, the identification of the imidazole amino acetic acid was not so definite as the identification of the histidine.

[118] Heffer, J., *Z. physiol. Chem.*, 1925, cxlv, 290.

[119] Fürth, O., *Biochem. Z.*, 1919, xcvi, 269.

[120] Hunter, G., *Brit. Med. J.*, 1922, ii, 751.

[121] Reinwein, H., *Deut. Arch. klin. Med.*, 1924, cxliv, 37. Reinwein, H., and Thielmann, F., *Arch. exptl. Path. Pharm.*, 1924, ciii, 115.

[122] Jaffé, M., *Ber. chem. Ges.*, 1874, vii, 1669; 1875, viii, 811.

[123] Siegfried, M., *Z. physiol. Chem.*, 1897-98, xxiv, 399.

[124] Swain, R. E., *Amer. J. Physiol.*, 1905, xiii, 30.

[125] Hunter, A., *J. Biol. Chem.*, 1912, xi, 537.

[126] Hunter, A., and Givens, M. H., *J. Biol. Chem.*, 1910, viii, 449.

[127] Barger, G., and Ewins, A. J., *J. Chem. Soc.*, 1911, xcix, 2336.

afford any dependable basis for relating this compound to normal histidine metabolism. Its structural relation to histidine, however, is evident, and its demonstrated formation from histidine by bacteria of the *Coli typhus* group, reported by Raistrick,[128] points to a close biological relation of urocanic acid and histidine. Raistrick suggested that its appearance in the urine of dogs is probably due to its formation from histidine by microörganisms in the large intestine.

Kotake and Konishi [129] observed that when dogs were fed large amounts of histidine (10 to 12 grams daily), urocanic acid was always present in the urine, though no other imidazoles could be detected. They were unable to find urocanic acid in the urine after the administration of imidazole lactic acid. The subcutaneous injection of histidine was also effective in producing urocanic acid, so that its formation in the metabolism of histidine appears to have been demonstrated.

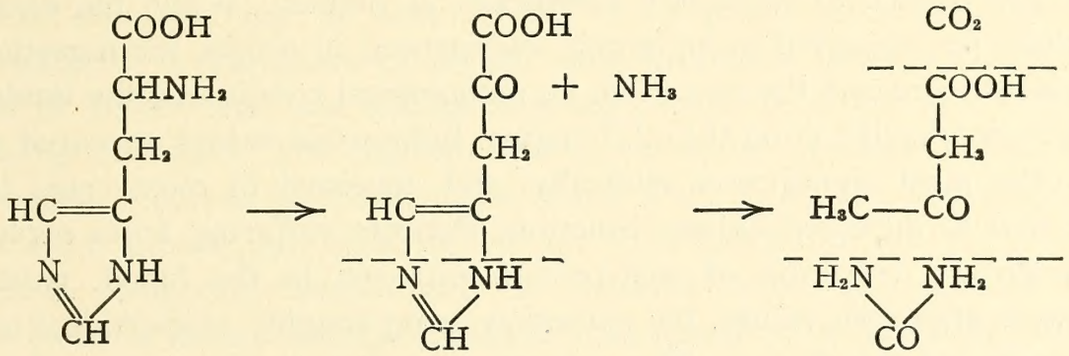
Pursuing the same problem further, Konishi [130] demonstrated a formation of acetoacetic acid in the perfused liver of a dog, when either urocanic acid, imidazole lactic acid, or histidine was added to the perfusing fluid. In fact, larger amounts of acetoacetic acid were obtained from the two former compounds than from histidine itself. Dakin and Wakeman [131] had previously shown that histidine does not yield acetoacetic acid readily under these conditions, while Dakin [132] was even less successful in later work, both in demonstrating acetoacetic acid formation in the dog liver, and glucose formation in phlorhizinized dogs. However, the work of Konishi and of Dakin and Wakeman appear to justify the conclusion that histidine is a precursor of acetoacetic acid.

In later work, Konishi demonstrated that urocanic acid is completely oxidized when administered orally to dogs,[133] that it is not produced from *d*-histidine, although the latter appears to be readily oxidized in the body,[134] and that imidazole propionic acid, as well as imidazole lactic acid, is not a precursor of urocanic acid.[135] Of the imidazole propionic acid administered in 5-gram doses subcutaneously about 10 per cent was excreted unchanged, along with an oxidation product that appeared to be hydroxy-imidazole propionic acid. In spite of the undoubted close association of imidazole lactic acid and histidine in metabolism, the administration of the former, either orally or subcutaneously to a dog in 10 to 12 gram doses, occasioned a loss of 50 to

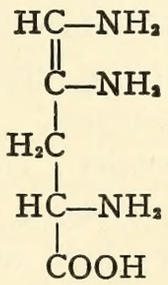
[128] Raistrick, H., *Biochem. J.*, 1917, xi, 71.
[129] Kotake, Y., and Konishi, M., *Z. physiol. Chem.*, 1922, cxxii, 230.
[130] Konishi, M., *Z. physiol. Chem.*, 1922, cxxii, 237.
[131] Dakin, H. D., and Wakeman, A. J., *J. Biol. Chem.*, 1912, x, 499.
[132] Dakin H. D., *J. Biol. Chem.*, 1913, xiv, 321.
[133] Konishi, M., *Z. physiol. Chem.*, 1925, cxliii, 181.
[134] Konishi, M., *Z. physiol. Chem.*, 1925 cxliii, 189.
[135] Konishi, M., and Tani, Y., *Z. physiol. Chem.*, 1925, cxliii, 193.

60 per cent in the urine. This evidence of a poor utilization of administered imidazole lactic acid in metabolism is confirmed by the work of Leiter,[136] who used much smaller doses (1 gram) and injected intravenously. Both the work of Leiter and of Konishi points to the fact that histidine is much more completely and readily oxidized in the body than is imidazole lactic acid. A close metabolic relation between histidine and imidazole lactic acid, and between histidine and urocanic acid seems to have been definitely established by the experiments of Kotake and Konishi, and of Konishi.

Reasoning analogously from the apparent method of formation of acetoacetic acid from phenylalanine and tyrosine, Dakin and Wakeman picture the cleavage of the imidazole nucleus as follows:



On the other hand, Koessler and Hanke [137] picture a cleavage involving the liberation of amino groups, and the formation of amines of the type—



According to Clifford,[138] there is present in animal tissues (skeletal muscle and liver) a heat-stable substance, not extractable by water, which catalyzes the cleavage of the imidazole nucleus; the disappearance of imidazole groupings is accompanied by the appearance of free amino nitrogen.*

[136] Leiter, L., *J. Biol. Chem.*, 1925, lxiv, 125.
[137] Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1924, lix, 803.
[138] Clifford, W. M., *Biochem. J.*, 1923, xvii, 549.

* Both Edlbacher (*Z. physiol. Chem.*, 1926, clvii, 108) and György and Röthler (*Biochem. Z.*, 1926, clxxiii, 334) have recently shown that the liver is particularly active in the cleavage of histidine. Edlbacher claims that approximately two-thirds of the nitrogen of histidine is removable as NH_3 by an enzyme found in the liver and absent from a number of other organs tested. György and Röthler, in autolysis experiments with liver tissue, found that additions of histidine, above all other amino acids tested, induced a marked production of NH_3.

A study of the excretion of total imidazole complexes by Koessler and Hanke [137] has yielded very interesting results, although they shed little light upon the metabolism of histidine. The method used [139] depends upon the development of colors ranging from yellow to red as the result of the interaction of imidazole compounds and *p*-phenyldiazonium sulfonate in alkaline (Na_2CO_3) solution. Since the identity of the major part of the imidazole complexes of urine is not known, the colorimetric results obtained with urine after the removal of interfering substances have been expressed in terms of a mixture of equal parts by weight of histidine dichloride, imidazole acetic acid, imidazole propionic acid, and imidazole lactic acid. It was found from a considerable number of analyses that normal adults excrete daily from 120 to 220 mgms. of imidazole complexes as defined. While the excreted imidazoles appeared to be largely endogenous in origin, the ingestion of protein increased the excretion. In pathological conditions, the imidazole excretion varied from 0 to 227 mgms. Subnormal values appeared to be of the most significance clinically, and appeared in most cases to be related to impaired kidney function. Patients suffering from nephritis, showing a retention of non-protein nitrogen in the blood, gave the lowest imidazole values, the excretion being roughly proportional to the severity of the disease. The average normal excretion appeared to be about 150 mgms. daily. From the results obtained with pathological subjects, an imidazole excretion of less than 80 mgms. on an adequate diet may be taken to indicate a definite impairment of the excretory function of the kidney, while values below 40 mgms. "suggest a very unfavorable state of the secreting renal tissue in the sense of a partial loss of function through destruction."

Leiter,[136] working in Koessler's laboratory, has used the Koessler and Hanke method for the estimation of imidazoles in experiments on a dog designed to throw some light upon the metabolism of histidine. Leiter was unable to demonstrate the presence of imidazoles in the blood of normal dogs and men, although Abel [140] found histidine in the dialysate from the circulating blood of the dog, and Abderhalden [141] has reported the identification of histidine in the dialysate from beef blood; more recently Demjanowski [142] has also isolated this amino acid from blood. Blau [143] found that basic amino acids precipitable by phosphotungstic acid could be isolated from all but a very small minority of human blood samples, while in most bloods the basic amino nitrogen

[139] Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 497.
[140] Abel, J. J., *Science*, 1915, xliii, 135.
[141] Abderhalden, E., *Z. physiol. Chem.*, 1921, cxiv, 250.
[142] Demjanowski, S., *Z. physiol. Chem.*, 1922, cxxii, 93.
[143] Blau, N. F., *J. Biol. Chem.*, 1923, lvi, 861.

constituted from 20 to 60 per cent of the total. Basic amino nitrogen was also demonstrable in dog and beef blood.

Leiter's work on histidine demonstrates the completeness with which this amino acid is metabolized. After the injection (intravenously) of 5 grams of histidine dichloride, the histidine content of the blood rose to 45 mgms. per 100 cc., and then fell gradually to 15 mgms. at the end of two hours. No other imidazole was demonstrated in the blood during this interval. The increase in urinary imidazoles during the following 24 hours (mainly confined to the first two hours) could account for only about 150 mgms. of the injected histidine. Only histidine was identified in the urine of this period. Besides histidine, injections of methyl imidazole, imidazole lactic acid, and imidazole were given to the same dog. In doses of 0.5 to 1.0 gram, methyl imidazole was excreted to the extent of 30 per cent, imidazole lactic acid to the extent of 40 per cent, and imidazole to the extent of 93 per cent in the following 24 hours. The highest rate of excretion of these imidazole compounds occurred in the first two hours after injection. Throughout these experiments, no evidence of an interconversion of the four imidazole compounds used could be obtained from examinations of either blood or urine. Methyl imidazole was found to be distinctly toxic for dogs in doses of 50 mgms. per kilo body weight.

The Synthesis of Histidine in the Body

The results of many feeding experiments involving rations containing, in place of histidine, imidazole derivatives and other compounds with which it might be related, have been interpreted with reference to the possibility or otherwise of certain metabolic reactions involving this amino acid. While under certain conditions such an interpretation is justified, a common fault is the failure to realize the inherent limitations of the significance of the results of feeding experiments in which the amount of food consumed is inadequate for growth or even for maintenance. It seems clear that if an experimental ration supports growth over a considerable period of time it must be complete with respect to all indispensable dietary factors. The fact that growth was secured is *prima facie* evidence that the food intake was adequate. However, it does not appear to be generally recognized that, if subsistence on an experimental ration leads to a decline in weight, it is not justifiable to conclude that the ration is incomplete qualitatively, *unless it has been shown, by proper control experiments, that the amount of food consumed was adequate to support growth.* In the absence of this proof, the results are open to two interpretations, *i.e.,* that the ration is incomplete

and hence incapable of supporting growth no matter how much may be eaten, or that the food intake was so small that growth could not result even if the ration were complete. In other words, the body weight curves in such uncontrolled experiments are significant only when an increase in weight is obtained; a decrease in weight, or maintenance of weight, is of ambiguous significance. The essential correctness of these propositions cannot be impaired by the gratuitous assumption that inadequate food intakes associated with declines in weight only result when the ration is incomplete.

For these reasons, the experiments of Cox and Rose [144] concerned with the possibility of substituting purines, creatine or creatinine for histidine seem inconclusive. The conclusion offered by the authors of the report, *i.e.*, that "the reaction of purine synthesis from histidine is an irreversible one in the animal organism," has not been demonstrated, since the intake of food containing histidine was always greater than the intake of food containing these other compounds in place of histidine.

In a paper on the availability of synthetic imidazoles in supplementing diets deficient in histidine, Cox and Rose [145] obtained growth with rations containing imidazole lactic acid in place of histidine, but no growth with rations containing as substitutes either imidazole, 4-methylimidazole, 4-hydroxy-methylimidazole, 4-imidazole formaldehyde, 4-imidazole carboxylic acid, 4-imidazole acetic acid, β-4-imidazole propionic acid, or β-4-imidazole acrylic acid (urocanic acid). However, the average food intakes on the latter rations rarely exceeded 4 grams per day for rats weighing approximately 100 grams, so that in most if not all of these experiments there is room for suspicion that the amounts of food consumed would not have supported growth, regardless of the completeness or incompleteness of the ration. This suspicion receives considerable support from the fact that in two of the experiments in which rations containing histidine or imidazole lactic acid were fed, the experimental rats declined in weight when the average daily food intake fell below 4.0 grams. If one may accept the conclusion that the basal ration, containing traces of histidine at most, is incomplete, the experiments indicate definitely that histidine is an indispensable amino acid and that imidazole lactic acid may replace it in metabolism, but, further than this, we are unable to follow the authors in their interpretations.

At about the same time, experiments of a similar nature were

[144] Cox, G. J., and Rose, W. C., *J. Biol. Chem.*, 1926, lxviii, 769.
[145] *Ibid.*, 781.

reported by Harrow and Sherwin,[146] using similar rations and methods of experimentation. These investigators obtained evidence of successful substitution of histidine by imidazole lactic acid and also by imidazole acrylic (urocanic) acid, since, upon rations containing the latter in place of histidine, experimental rats were maintained at practically constant weight. The average results obtained with the different rations used are summarized in Table 27, from our own computations.

TABLE 27. *Summary of Experiments of Harrow and Sherwin.*

No. of Periods	Ration	Aver. Body Weight	Aver. Daily Food	Aver. Daily Gain
		gms.	gms.	gms.
8	Casein	93	6.9	+ 2.97
17	Basal, no histidine	99	2.3	— 0.86
6	Basal plus histidine	107	7.2	+ 1.67
4	Basal plus imid. lactic	98	5.9	+ 1.36
4	Basal plus imid. pyruvic	81	3.8	+ 0.45
4	Basal plus imid. acrylic	112	3.2	— 0.10
3	Basal plus imidazole	102	3.4	— 0.25

It appears that the changes in weight, although in prompt response to the changes in ration, are closely correlated with the amounts of food consumed, and hence, in the absence of any evidence to the contrary, may be the direct result of the variable food intake. This appears to be a possible interpretation, and cannot be refuted by any hypothesis of the causes operating in determining the amount of food consumed, causes concerning which we are in the most profound ignorance. The experiments cannot be said to demonstrate that the basal ration was inadequate, since the daily consumption of 2.3 grams of food, no matter how well balanced it may be, could not maintain a rat weighing 99 grams, and the response to the various additions made to the basal ration cannot be interpreted in unequivocal fashion. The interpretation of Harrow and Sherwin, in which the results are used in decisions concerning the intermediary metabolites of histidine, and the reversibility and relative speed of the reactions involved, represents an amazing disregard of the possible significance of the food intakes recorded. However, in defense of this interpretation, it may be said that it represents but the ultimate result of a line of reasoning that is implied and at least partially pursued in a considerable number of reports of feeding experiments in which no control of the food consumption is attempted.

THE RELATION OF HISTIDINE TO PURINE METABOLISM

The relation of histidine to purine metabolism, first indicated by the experiments of Ackroyd and Hopkins and later by those of Rose

[146] Harrow, B., and Sherwin, C. P., *J. Biol. Chem.*, 1926, lxx, 683.

and Cook, have been considered elsewhere (see page 258) in connection with the question of the interchangeability of histidine and arginine in metabolism. The supposed relation is based upon the fact that, on rations lacking histidine, the allantoin excretion of rats is lower than on rations containing histidine. However, this does not appear to constitute a demonstration that histidine is a precursor of the purine nucleus, since the purine excretion of animals is evidently affected by so many factors, apparently unrelated to purine synthesis.[147] The purine excretion seems to be the result of an unstable equilibrium between synthesis and catabolism, either of which may be subject to acceleration or depression. The final demonstration, therefore, that histidine is a precursor of the purine ring, must apparently be furnished by an experimental method whose results are more readily interpretable than those of the feeding experiments cited in this connection. The chemical mechanism of purine synthesis from histidine is not at all obvious. Many negative results[148] have been reported from experiments designed to establish a relation between histidine and purines in animal metabolism, but such results cannot be considered as invalidating the positive indications that have been observed.

Recapitulation

It is evident that intimate metabolic relations exist between histidine on the one hand and the corresponding hydroxyl, ketonic, and unsaturated acids that might result from its deamination. The work of Kotake and Konishi may be interpreted to mean that the unsaturated acid (urocanic acid) is not formed by way of the hydroxy acid, nor by way of imidazole propionic acid, while the feeding experiments of Cox and Rose and of Harrow and Sherwin indicate that the deamination of histidine is accomplished by reversible reactions. The experiments of Leiter and of Konishi demonstrate the ready metabolizability of histidine, as compared, for example, with one of its demonstrated metabolites, imidazole lactic acid. The greater ease of oxidation of imidazole propionic acid than of imidazole lactic acid, revealed by the experiments of Konishi, is difficult to reconcile with prevailing ideas of deamination. It is clear from this work that the flooding of the tissues with substances that, as they are produced in metabolism, are readily and completely utilized, may result in a very poor utilization, so that conclusions from such injection experiments have only a quali-

[147] Rose, W. C., *Physiol. Revs.*, 1923, iii, 544.

[148] Abderhalden, E., and Einbeck, H., *Z. physiol. Chem.*, 1909, lxii, 322; Abderhalden, Einbeck and Schmid, *ibid.*, 1910, lxviii, 395; Lewis, H. B., and Doisy, E. A., *J. Biol. Chem.*, 1918, xxxvi, 1; Stewart, C. P., *Biochem. J.*, 1925, xix, 266.

tative application to the reactions occurring spontaneously within the tissues. The rôle of histidine as a purine precursor, suggested by the experiments of Ackroyd and Hopkins, Rose and Cook, and Stewart, requires investigation by other methods amenable to more definite interpretation.

Carnosine

Prominent among the extractives of muscle tissue is the base carnosine. The discovery of this base was reported by Gulewitsch and Amiradzibi [149] in 1900. Eleven years later [150] Gulewitsch demonstrated that carnosine was a dipeptide consisting of histidine and β-alanine, while in 1918 the final step in its identification was effected independently by Barger and Tutin [151] and Baumann and Ingvaldsen.[152] It was found that hydrolysis of deaminocarnosine yielded unchanged histidine, indicating that carnosine is β-alanyl-histidine, rather than histidyl-β-alanine. This conclusion was established definitely by the synthesis of β-alanyl-histidine and by determination of its chemical properties, optical rotation, and its elementary composition, all of which were identical with those of carnosine. A recent study of carnosine and its chemical compounds has been reported by Smorodinzew.[153]

Carnosine, like creatine, occurs in greatest amounts in skeletal muscle. Smorodinzew [154] found 0.182 per cent of carnosine in horse flesh, 0.265 per cent in ox flesh, 0.096 per cent in sheep flesh, 0.289 per cent in swine flesh, and 0.164 per cent in human flesh.[155] Heart muscle contains considerably less carnosine than skeletal muscle, according to Clifford,[156] while Smorodinzew [157] found no carnosine in liver, and Dem'yanovskii [158] none in spleen. Hunter [159] reports the occurrence of small amounts of carnosine (1 to 2 mgms. per 100 cc.) in ox blood.

In a study of the distribution of carnosine in the animal kingdom, Clifford reported specific differences in the carnosine content of skeletal muscle. Separate estimations were made upon the red and white skeletal muscle of the rat, rabbit and domestic fowl, but no significant differences were found. All invertebrate muscle examined (sea anemone, crayfish, spider, snail, oyster, mussel, scallop) appeared to be free

[149] Gulewitsch, W., and Amiradzibi, S., *Z. physiol. Chem.*, 1900, xxx, 565; *Ber. chem. Ges.*, 1900, xxxiii, 1902.
[150] Gulewitsch, W., *Z. physiol. Chem.*, 1906-07, 1, 535; 1911, lxxiii, 434.
[151] Barger, G., and Tutin, F. *Biochem. J.*, 1918, xii, 402.
[152] Baumann, L., and Ingvaldsen, T., *J. Biol. Chem.*, 1918, xxxv, 263.
[153] Smorodinzew, I. A. *Russ. Physiol. J.*, 1919, ii, 285; *Chem. Abst.*, 1921, xv, 2646.
[154] Smorodinzew, I. A., *Z. physiol. Chem.*, 1922, cxxiii, 116.
[155] Smorodinzew, I. A., *J. Russ. Phys. Chem. Soc.*, 1917, xlix, 1, 263; *Chem. Abst.*, 1923, xvii, 3049.
[156] Clifford, W. M., *Biochem. J.*, 1921, xv, 725.
[157] Smorodinzew, I. A., *Z. physiol. Chem.*, 1914, xcii 214.
[158] Dem'yanovskii, S., *Russ. J. Physiol.*, 1922, iv, 193; *Physiol. Abst.*, vii, 401.
[159] Hunter, G., *Biochem. J.*, 1925, xix, 34.

from carnosine. Among the fishes, reptiles, and birds examined there was some indication that certain of the zoölogical families in each class contain no carnosine, while others always contain it. Among the mammals, none thus far examined among twelve species was found to be carnosine-free. That the nature of the food supply of the animal is not an important factor in determining the carnosine content of muscle is shown by the fact that the carnivorous leopard and the herbivorous antelope showed almost the same percentage of carnosine, while the bull and the sheep, subsisting upon similar food, show a great difference, bull muscle containing almost three times as much carnosine as sheep muscle (see also the analyses of Smorodinzew cited above). The cold storage of muscle was found to lower decidedly the carnosine content.[160] Clifford used in her work a modification [161] of the colorimeter method of Koessler and Hanke for the determination of imidazoles. Clifford's method has been criticized and modified by Hunter,[162] who admits, however, that his method is not entirely satisfactory, since apparently some other substance than carnosine contributes to the diazo value.

In opposition to the constant values reported by Clifford for the carnosine content of the muscles of any one species of animals, Mitsuda [163] and Hunter [159, 164] have obtained widely varying results for the same muscle of different cats, and still greater differences among different muscles. Mitsuda was unable to detect any effect of sectioning of the motor nerve or of the condition of decerebrate rigidity upon the carnosine content of muscle. However, Hunter was able to lower enormously the carnosine content of white striated muscle (gastrocnemius and plantaris) by protracted fasting; the red muscle (soleus) was not appreciably affected. By refeeding with meat, the low values resulting from starvation could be raised to normal. In Hunter's observations, the red muscle always contained low and fairly constant amounts of carnosine.

The fact that carnosine contains an amino acid, β-alanine, not found in proteins and hence in all probability a specific synthetic product of the animal cell, suggests strongly that the base performs a specific function in the body, either in the muscle itself, or, as a hormone, in the regulation of other tissues, or possibly both. Carnosine cannot reasonably be classed as an intermediate product or an end-product of metabolism, particularly since, according to Baumann and Ingvaldsen,[152] it is not hydrolyzed by extracts of either muscle or liver, which were

[160] Clifford, W. M., *Biochem. J.*, 1922, xvi, 341.
[161] Clifford, W. M., *Biochem. J.*, 1921, xv, 400.
[162] Hunter, G., *Biochem. J.*, 1921, xv, 689.
[163] Mitsuda, T., *Biochem. J.*, 1923, xvii, 630.
[164] Hunter, G., *Biochem. J.*, 1924, xviii, 408.

active in hydrolyzing another dipeptide, glycyl-tryptophane. However, experimental attempts to demonstrate its physiological function have not been notably successful. The physiological effects of carnosine injection in animals are similar to, if not identical with, those of histamine, though larger amounts of carnosine are required for the same result.[165] Experiments by Krimberg [166] led him to ascribe to carnosine the typical properties of a hormone possessing a powerful stimulating effect upon gastric secretion. This effect of carnosine was later confirmed by Komarow [167] and by Krimberg and Komarow,[168] but Schwarz and Goldschmidt [165] were not able to obtain any effect of carnosine injection upon the secretion of digestive juices. The cleavage of carnosine by intestinal bacteria has been studied by Hefter [169] in an attempt to establish a relation between the dipeptide and intestinal intoxication.

Apparently the precise physiological function of carnosine, as of the numerous other nitrogenous extractives of tissues, is a problem for the future to solve. Are these substances acting *in situ* in the regulation of tissue functioning, or are they hormones concerned with the regulation of other tissues in which they are not produced, or are they temporary forms of storage of certain important chemical compounds or chemical radicles to be used later in the elaboration of hormones? Hunter's data indicating a dependence of the carnosine content of red muscle upon the food supply would appear to favor the latter type of function for this dipeptide.

Histamine

It is a fact of great significance that histidine, a substance entirely indifferent pharmacodynamically,[170] should be transformable by simple decarboxylation into such an active substance as histamine. Histamine is one of the most powerful depressor and oxytocic substances known. When injected rapidly in minute quantities (1 mg. per kgm. body weight) into etherized animals, histamine soon reduces the arterial pressure to the shock level of 30 to 40 mm. Hg., by causing an apparent oligemia due to the stagnation of blood in capillaries and venules that have been greatly dilated as the result of the direct action of the drug in abolishing their tone.* Histamine causes capillary dilatation, and

[165] Schwartz, C., and Goldschmidt, E., *Arch. ges. Physiol.*, 1924, ccii, 435; McClintock, J. T., and Hines, H. M., *Proc. Soc. Expt. Biol. Med.*, 1925, xxii, 515.
[166] Krimberg, R., "Zur Frage über den Mechanismus der Magensekretion," Charkow, 1915.
[167] Komarow, S. A., *Biochem. Z.*, 1924, cli, 467.
[168] Krimberg, R., and Komarow, S. A., *Biochem. Z.*, 1926, clxxi, 169.
[169] Hefter, J., *Z. physiol. Chem.*, 1925, cxlv, 276.
[170] Guggenheim, M., and Löffler, W., *Biochem. Z.*, 1916, lxxii, 303; Hirschfelder, A. D., and Comtwell, W., *J. Pharm.*, 1918, xi, 178.
* In the rabbit, however, histamine, in certain doses at least, induces a pressor effect.

at the same time arteriole constriction.[171] The oxytocic power of histamine is such that a concentration of one in 250 million parts will still stimulate a strip of guinea pig uterus. Subcutaneous or intravenous injection of histamine exerts a temporary antidiuretic effect, similar to, though distinct from, that of pituitrin.[172] Ivy and Javois [173] have demonstrated that histamine is a potent gastric stimulant when given to animals by stomach tube, intravenously, or subcutaneously in proper doses. Other amino acids than histidine produce physiologically active amines upon decarboxylation, but none of these amines are as active as histamine.

Although these pharmocological properties of histamine are extremely interesting, they have no direct relation to histidine metabolism except in so far as histamine is produced in the animal organism. Its production from histidine involves removal of the carboxyl group, and, as early as 1910, was shown by Ackermann [174] to result from the action of certain unidentified bacteria (obtained from putrefying meat) on histidine hydrochloride. Mellanby and Twort,[175] a few years later, isolated from the intestinal contents of the guinea pig a bacillus of the colon typhoid group that possessed the capacity of converting histidine into histamine, while in the same year Berthelot and Bertrand [176] obtained from the human intestine a bacillus, possessing many of the characteristics of Friedländer's pneumobacillus, that could also decarboxylate histidine.

A systematic study of the production of histamine by the bacilli belonging to the colon group was reported by Koessler and Hanke [177] in 1919, and similar studies by the same investigators were described later.[178] It was found that the human intestinal tract is normally inhabited by microörganisms that have the faculty of decarboxylating histidine to histamine and tyrosine to tyramine, and probably other amino acids as well. This amine production (carboxylase activity) was shown to be a specific function of certain species of bacteria and even of certain strains within the species. The carboxylase activity apparently is called into play only when the medium acquires a strong acid reaction. Apparently the amines are formed by the bacterial cell to neutralize an acidity in excess of that compatible with its normal life processes. Contrary to many statements in the literature to the effect that carbo-

[171] Dale, H. H., and Richards, A. N., *J. Physiol.*, 1918-19, lii, 110; Dale and Laidlaw. P. P., *ibid.*, 1918-19, lii, 355. See also Hiller, A., *J. Biol. Chem.*, 1926, lxviii, 833, 847.
[172] Moletor, H., and Pick, E. P., *Arch. exptl. Path. Pharm.*, 1924, ci, 198.
[173] Ivy, A. C., and Javois, A. J., *Amer. J. Physiol.*, 1925, lxxi, 604.
[174] Ackermann D., *Z. physiol. Chem.*, 1910, lxv, 504.
[175] Mellanby, E., and Twort, F. W., *J. Physiol.*, 1912-13, xlv, 53.
[176] Berthelot, A., and Bertrand, F. M., *Compt. rend. Acad.*, 1912, cliv, 1643, 1826.
[177] Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 539.
[178] Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1924, lix, 835, 855.

hydrates prevent the formation of histamine from histidine, Koessler and Hanke found that histamine is never formed except in the presence of an easily available source of carbon, such as glycerol or glucose. Colon bacilli can be divided into three classes with respect to their carboxylase activity on histidine and tyrosine, namely, (*a*) those that form tyramine from tyrosine, (*b*) those that form histamine from histidine, and (*c*) those that do not have the faculty of decarboxylating either of these amino acids. Thus, bacteria that decarboxylate histidine do not decarboxylate tyrosine, and *vice versa.*

Since histidine-containing proteins are always present in a normal diet and since decarboxylating bacteria are normally found in the intestinal tract, it follows that histamine (and other amines) must necessarily be normal constituents of the contents of the lower levels of the intestine. Meakins and Harington [179] demonstrated the presence of a substance in the contents of the human ileum and cecum that possessed similar pharmacological and chemical properties to histamine, and Hanke and Koessler [180] were able to offer more definite chemical proof of the occurrence of histamine (sometimes in considerable amounts) in the cecal contents and in the feces of human subjects and in dog feces, though none was found in the intestinal contents of guinea pigs. Gerard [181] found histamine present in the contents of isolated closed loops of either the small or the large intestine of dogs. The occurrence of histamine in the intestinal mucosa, first demonstrated by Barger and Dale [182] and later confirmed by Abel and Kubota [183] and Gerard, is referable, in all probability, to the septic production of this base within the intestinal lumen.

It is a matter of profound importance to the science of pathology that such physiologically active compounds as histamine are being constantly produced in the gastro intestinal tract in such amounts that, if any considerable absorption occurred, definite disturbances in blood pressure, smooth muscle control, and in other functions would be expected. In fact, attempts have been made to trace many disease conditions referable to the bowel to the amines therein continually produced. Thus, the symptoms of intestinal intoxication and obstruction, bronchial asthma, and urticaria have been labeled as histamine disorders, with what success the authors of this review do not feel competent to judge. Presumably the evidence is more circumstantial than direct, but the multiplicity of these possible untoward effects of histamine warrants

[179] Meakins, J., and Harington, C. R., *J. Pharm. and Exp. Therap.*, 1921, xviii, 455.
[180] Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1924, lix, 879.
[181] Gerard, R. W., *J. Biol. Chem.*, 1922, lii, 111.
[182] Barger, G., and Dale, H. H., *J. Physiol.*, 1910-11, xli, 499.
[183] Abel, J. J., and Kubota, S., *J. Pharm. and Exp. Therap.*, 1919, xiii, 243.

a consideration of the defenses the animal organism possesses against the continual inflow of proteinogenous amines into the systemic circulation.

However, before considering the possible detrimental effects on the organism of histamine production in the bowel, it is interesting to note the suggestions of Ivy and Javois [173] of a possible favorable effect. According to these investigators, the proteinogenous amines formed in the intestine, prominent among which is histamine, may well serve as normal gastric secretory excitants, accounting in part, at least, for the intestinal phase of gastric secretion and playing a rôle in the genesis of the continuous secretion of the gastric glands. Besides demonstrating a definite stimulatory effect of histamine and a number of other amines upon the gastric glands, it was also shown in support of this theory that drastic catharsis usually depresses the continuous secretion of gastric juice. The chemical and physiological similarities of histamine and gastrin have been considered suggestive by Koch, Luckhardt and Keeton,[184] though their chemical identity has not been postulated. Parsons [185] has shown that histamine, though present in the intestinal mucosa and possessing a secretagogue action on the pancreas, is much less potent in this respect than secretin, and for other reasons also cannot be considered identical with this hormone.

That the presence of considerable amounts of histamine in the intestinal tract may be quite innocuous has been repeatedly shown. Oehme [186] observed no symptoms of histamine intoxication after giving 0.5 gram of the amine to rabbits by mouth. Ivy and Javois [173] gave 0.75 gram to dogs with no apparent effect, while Koessler and Hanke [187] also observed no reaction in a 5 kg. dog after the administration of 0.5 gram by stomach. A guinea pig can withstand 0.1 gram of histamine dichloride in the stomach with only mild symptoms (Koessler and Hanke), and a man responds with only occasional vomiting to the ingestion of 0.225 gram of the base (Ivy and Javois).

Furthermore, the absorption or disappearance of administered histamine from the intestinal tract is rapid. Mellanby,[188] working with cats, found that the rate of disappearance of histamine from the intestine increases from duodenum to cecum, while Meakins and Harington,[189] measuring the rate of absorption by the rate of fall of blood pressure produced, observed that absorption is greatest from the ileum, somewhat less from the duodenum, and very much less, though still perfectly

[184] Koch, F. C., Luckhardt, A. B., and Keeton, R. W., *Amer. J. Physiol.*, 1920, lii, 508.
[185] Parsons, E., *Amer. J. Physiol.*, 1924-25, lxxi, 479.
[186] Oehme, C., *Arch. exptl. Path. Pharm.*, 1913, lxxii, 76.
[187] Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1924, lix, 889.
[188] Mellanby, E., *Quart. J. Med.*, 1916, ix 165.
[189] Meakins, J., and Harington, C. R., *J. Pharm. and Exper. Therap.*, 1922-23, xx, 45.

definite, from the cecum and stomach. Histamine is rapidly absorbed from the mucous membrane of the mouth.

Koessler and Hanke observed a disappearance of over half of the histamine administered to either dogs or guinea pigs within 2 hours. It was not destroyed by the microörganisms residing within the intestines; in one of the cases, at least, removal by defecation did not occur, and hence it was most likely absorbed. In fact, small amounts of histamine, presumably portions of that administered, were found in the intestinal mucosa, and even in the liver, so that *some* of the amine appears to have been absorbed as such. If all the histamine that disappeared from the intestinal tract was absorbed and thrown into the systemic circulation, it may be calculated that its entrance into the blood would have been at the approximate rate of 0.5 mgm. per minute for the guinea pig and 2.2 mgms. per minute for the dog. However, such a rate of injection will kill the guinea pig and probably the dog also, though as a matter of fact, the guinea pig became only mildly ill, while the dog was unaffected. Hence, detoxication of the absorbed histamine must have occurred.

That detoxication of histamine undoubtedly occurs in the animal organism was also proved by Oehme,[186] who showed that many times the lethal dose of histamine might be injected into dogs with no serious effect provided the rate of injection was slow.

The glandular organ ordinarily presumed to bear the main burden of the detoxication of intestinal poisons is the liver. Meakins and Harington, from studies on Eck fistula dogs, believed that the liver exerts some protective action against heavy dosages of histamine, though the effect was assumed to be more of a mechanical than of a chemical nature, since no actual destruction of histamine in the liver could be demonstrated. Oehme, also, believed that the liver was effective in this respect. Ewins and Laidlaw[190] have shown that the liver actively deaminates and oxidizes tyramine and indole-ethylamine, converting them into the corresponding substituted acetic acids, but Dale and Laidlaw[191] were unable to demonstrate the production of imidazole acetic acid from histamine in perfusion experiments on surviving livers, though they obtained some evidence of its disappearance; however, the extent of destruction was not considerable, and it was evident that the liver could not effectively detoxicate this particular amine. Meakins and Harington, in similar experiments, confirmed the refractory behavior of histamine to detoxication in the liver.

[190] Ewins, A. J., and Laidlaw, P. P., *J. Physiol.*, 1910, xli, 78; *Biochem. J.*, 1913, vii, 18.
[191] Dale, H. H., and Laidlaw, P. P., *J. Physiol.*, 1910, xli, 318; *ibid.*, 1911, xliii, 182.

A most important study of the detoxication of proteinogenous amines in the animal organism was made by Guggenheim and Löffler,[192] who found that isoamylamine, phenylethylamine, *p*-hydroxyphenylethylamine, and indolethylamine are rapidly detoxicated after injection, in accordance with the previous observations of Ewins and Laidlaw. Again, however, histamine proved refractory, since no imidazole acetic acid could be isolated from the urine, and, in liver perfusion experiments, no appreciable destruction of histamine could be demonstrated by biological methods.

The inability of the liver to detoxicate histamine effectively is indicated clearly by less direct experiments. Popielski [193] observed the usual symptoms after injecting histamine into the mesenteric veins, from which it must pass through the liver before entering the systemic circulation. Ivy and Javois [173] found Eck fistula dogs to be as tolerant as normal dogs to doses of 0.5 gram to 0.75 gram of histamine administered by stomach tube, while Koessler and Hanke [187] determined that the effect of injected histamine is reduced somewhat if it has to pass through a capillary network before reaching the general circulation, but that it is immaterial whether the capillary system is in the lower extremity or in the liver. Hence, the liver cannot be considered an effective or specific organ for the detoxication of histamine, though it can, apparently, handle other proteinogenous amines in a highly effective manner.

The conclusion of Koessler and Hanke, therefore, that "histamine may be rendered pharmacologically inert in its passage through the wall of the intestine" seems to be justified, though no direct evidence of such a function of the intestinal mucosa has been advanced. If this conclusion proves to be correct, the intestinal tract is itself able to render innocuous this highly toxic product of intestinal bacterial action, so that only when the intestinal wall is injured, therefore, is it likely that histamine intoxication will result. Against the conclusion it may be said that the analytical data on which estimates of the extent and rate of disappearance of ingested histamine from the intestines are based are not wholly satisfactory. Only a 60 per cent recovery of histamine from intestinal and fecal material is claimed for the analytical method, and how variable this percentage may be is unknown. The possibility exists that, at least for histamine produced within the intestinal tract, the normal method of disposal is excretion from the bowel in the feces. The possibility of the adsorption of histamine by the solid

[192] Guggenheim, M., and Löffler, W., *Biochem. Z.*, 1915-16, lxxii, 325.
[193] Popielski, L., *Arch. ges. Physiol.*, 1920, clxxviii, 237.

constituents of the contents of the lower bowel is admitted by Koessler and Hanke. It is of significance in this connection that absorption of histamine appears to be least rapid in that segment of the alimentary canal where it would normally be produced, *i.e.*, the cecum and colon.

While the significance and the fate of histamine produced within the gastro-intestinal tract by bacterial action have received much consideration from investigators, it is a matter of great importance to inquire also concerning the possibility of the production of histamine in metabolism. It has been frequently noted that extracts of a wide variety of animal tissues possess a marked depressor and vasodilator action on the circulation, and also a secretin-like effect upon the pancreas and the intestinal glands, and frequently a stimulating effect upon smooth musculature. Furthermore, the vasodilatation occurring in muscles and glands during functional activity appears to be in part at least a direct effect of some chemical substance produced in metabolism; also when a large mass of tissue is injured seriously by trauma, a symptom-complex involving preëminently vasodilatation and a marked lowering of blood pressure supervenes unless the blood supply to the injured area is effectively restricted.

These effects of tissue extracts on functional activity, and traumatic shock are similar to the effects of histamine and have led some authors to assume a wide distribution in the body of histamine or of substances closely allied with it. Apparently the first investigators to call attention to these similarities were Dale and Laidlaw,[191] though they did not go so far as to assume that these effects were due to the universal presence of histamine in animal tissues. However, Abel and Kubota [183] have argued at length for such a simple and attractive explanation of these diverse phenomena and many others offering points of similarity. They suggest that histamine plays an important rôle in the animal organism as a stimulant for the gastric and intestinal musculature and also as a dilator of capillaries in the gastric and intestinal walls during digestion and in all organs during periods of increased activity. They also believe that histamine is the most powerful depressant originating in mutilated tissues, and they suggest that it plays the leading rôle among the chemical factors concerned in traumatic shock. They believe that histamine is produced by proteolysis, thus accounting for its wide occurrence in animal tissues, and that its presence in peptone preparations adequately explains the symptoms of peptone shock. Furthermore, it is pointed out that the protein poisons of Vaughan possess histamine-like effects on the body, a fact recognized by Vaughan himself. Finally, the similarity between the symptoms of histamine shock and of

anaphylactic shock after protein sensitization is given its most obvious interpretation.

The whole picture presented by Abel and Kubota is attractive because of its simplicity, but unfortunately its experimental justification is yet to be attained. It is true that Abel and Kubota offer chemical and pharmacological evidence of the occurrence of histamine in liver, muscle, washed gastric and intestinal mucosa, and in a dried commercial preparation of the posterior lobe of the pituitary body, and of the production of histamine by the hydrolysis of proteins and of its presence in ereptone and Witte's peptone. However, actual isolation and identification of histamine was not accomplished in any case, although the evidence obtained of its occurrence in the pituitary and in the gastric and intestinal mucosa seems convincing. With the other tissues and substances, it was shown simply that basic compounds, showing several of the solubilities and precipitabilities of histamine, as well as its most prominent pharmacological properties, were present.*

It was thought by Abel and Kubota that the occurrence of histamine in the posterior lobe of the pituitary body accounted for the plain-muscle-stimulating and depressor properties of this tissue, but in the following year Abel and Nagayama [194] isolated another substance, much more active than histamine in oxytocic properties, which they now consider to be the active hormone of this gland. They still believe that histamine is always associated with this hormone, and accounts in part, at least, for the depressor action of pituitary extracts. In this they are opposed by Hanke and Koessler,[195] who were unable to find histamine in fresh glands and ascribe the positive findings of Abel and Kubota to putrefactive changes in the commercial product investigated by them, and by Dale and Dudley,[196] who believe that, if histamine occurs at all in the hypophysis, it is in such slight traces that its chemical identification is impracticable. However, Roca,[197] working in Abel's laboratory, has obtained much the same sort of evidence (though less convincing) of the occurrence of histamine in fresh glands as that previously offered by Abel and Kubota.

The occurrence of histamine as a product of proteolysis seems improbable, if only because its presence in protein hydrolysates has never before been noted by the many investigators concerned with protein

* Best, Dale, Dudley and Thorpe (*J. Physiol.*, 1927, lxii, 397), however, have recently reported the isolation of histamine from a number of fresh animal tissues, lung tissue especially giving relatively large amounts, and later Thorpe reported its isolation from muscle (*Biochem. J.*, 1928, xxii, 94). This work appears to be a striking confirmation of the general argument of Abel and Kubota.

[194] Abel, J. J., and Nagayama, T., *J. Pharm. and Exptl. Therap.*, 1920, xv, 347.
[195] Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1920, xliii, 557.
[196] Dale, H. H., and Dudley, H. W., *J. Pharm. and Exptl. Therap.*, 1921-22, xviii, 27.
[197] Roca, J., *J. Pharm and Exptl Therap.*, 1921-22, xviii, 1.

chemistry. The unsatisfactory evidence offered for its occurrence in proteins by Abel and Kubota also inclines one to the belief that the negative results of Hanke and Koessler [198] in attempting to confirm their work are of the greater significance. While failing to find any evidence of the presence of histamine in proteins, Hanke and Koessler did find that a substance pharmacologically similar to histamine can be split from casein by acid hydrolysis.

In this connection a suggestion of Arai [199] seems pertinent. Since the amino acids, in particular histidine, become pharmacologically active upon removal of their carboxyl group, it appeared reasonable to Arai to expect that compounds of amino acids involving the carboxyl group should exhibit amine-like physiological effects. As a matter of fact, he found that the chlorhydrate of histidine methyl ester possessed a similar, though less potent, effect on blood pressure and smooth musculature as histamine itself. Other amino acid esters investigated were *l*-tyrosine ethyl ester, *dl*-phenylalanine ethyl ester, *l*-leucine ethyl ester, *l*-cystine ethyl ester, and glycine ethyl ester. These esters were all more or less active pharmacodynamically, although the corresponding amino acids are inactive. It is pointed out by Arai that proteins and their derivatives are known to consist of combinations of amino acids. The coupling of the individual units in general seems to be of the acid amide type. On the other hand, an ester-like coupling between carboxyl and hydroxyl groups is not impossible. In view of the above experimental results, the thought is suggested that indifferent amino acids could become toxic as a result of the manner of their linking together. It is not improbable that toxic proteins or toxic products of incomplete proteolysis are thus formed, and that the anaphylactic reaction may be related to such products.

The suggestion of Arai seems to be a fertile one, and may ultimately explain many of the phenomena that Abel and Kubota have attempted to explain in terms of histamine. It may be possible also that other unions of amino acids than that suggested by Arai may so mask the effect of carboxyl groups as to produce amine-like properties in the conjugated products.

There is another possibility that may lead to the production of histamine in metabolism, *i.e.*, the decarboxylation of histidine. The capacity of animal tissues to decarboxylate organic acids is not one that has been frequently observed or that is generally recognized. In 1911 Neuberg and Karczag [200] discovered that yeasts decompose pyruvic

[198] Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1920, xliii, 543.
[199] Arai, M., *Biochem. Z.*, 1923, cxxxvi, 202.
[200] Neuberg, C., and Karczag, L., *Biochem. Z.*, 1911, xxxvi, 60, 68, 76.

acid into acetaldehyde with the evolution of CO_2, and in later reports,[201] Neuberg and his coworkers have shown that this reaction is of fundamental importance in the alcoholic fermentation of sugars. Furthermore, as Shaffer [202] points out, "there has gradually been accumulated a train of evidence which has been interpreted in support of the idea that glucose metabolism in the animal body follows much the same path as in fermentation and that pyruvic acid and acetic aldehyde are in the animal body also obligate intermediates." While Shaffer himself is not inclined to consider either pyruvic acid or acetaldehyde as obligate glucose metabolites, he cites considerable evidence favoring the view that acetaldehyde may be formed from glucose through pyruvic acid. In the case of yeast fermentation, the decarboxylation of pyruvic acid has been ascribed to an enzyme "carboxylase," so that the question at issue may be considered as relating to the occurrence of carboxylases in animal tissues.

In 1912, Tschernorutzky [203] found that muscle and liver hash (brei) of dogs and rabbits possessed a considerable capacity for destroying pyruvic acid under antiseptic conditions, though the products of the reaction were not identified, while Maeda [204] observed the same to be true for human placental tissue. Mayer [205] obtained very definite evidence that oxalyl acetic acid in properly buffered solutions was decarboxylated completely by liver tissue from dogs and swine, giving an amount of carbon dioxide corresponding to the production of pyruvic acid. The reaction did not depend upon the presence of oxygen.

Neuberg and Gottschalk,[206] in experiments with liver and muscle brei, have found that acetaldehyde is produced in quantitatively determinable amounts, and that the rate of its production is increased by the addition of carbohydrates (glycogen, glucose, hexose monophosphate, fructose) and probable carbohydrate metabolites (glyceric aldehyde, dioxyacetone, glycolaldehyde). Of these added carbohydrates and carbohydrate derivatives, glycogen was the most readily convertible into acetaldehyde. Furthermore, the production of acetaldehyde in liver and muscle brei with no carbohydrate additions was increased two and one-half times by the addition of insulin, confirming an earlier observation of Neuberg, Gottschalk, and Strauss.[207] In an extension of these investigations, Gottschalk [208] found that, under aërobic conditions, liver

[201] Neuberg, C., and Färber, E., *Biochem. Z.*, 1916, lxxviii, 238; Neuberg and Reinfurth, E., *ibid.*, 1918, lxxxix, 365; 1920, cvi, 281; Neuberg and Nord, F. F., *ibid.*, 1919, xcvi, 133.
[202] Shaffer, P. A., *Physiol. Revs.*, 1923, iii, 394.
[203] Tschernorutzky, M., *Biochem. Z.*, 1912, xliii, 486.
[204] Maeda, K., *Biochem. Z.*, 1923, cxliii, 347.
[205] Mayer, P., *Biochem. Z.*, 1914, lxii, 462.
[206] Neuberg, C., and Gottschalk, A., *Biochem. Z.*, 1924, cxlvi, 164, 182.
[207] Neuberg, C., Gottschalk, A., and Strauss, H., *Deutsch. med. Woch.*, 1923, xlix, 1407.
[208] Gottschalk, A., *Biochem. Z.*, 1924, cxlvi, 582.

brei (rabbit) produced acetaldehyde in small amounts, and that the addition of pyruvic acid increased the rate of aldehyde production 90 and 160 per cent in two experiments. Under anaërobic conditions, the liver brei itself produced no trace of acetaldehyde, while determinable amounts were produced following the addition of pyruvic acid.

These experiments of Neuberg and Gottschalk appear to demonstrate that liver (and probably muscle) tissue possesses the capacity of decarboxylating pyruvic acid; possibly due to the presence of a carboxylase. How specific this decarboxylating action is cannot at present be judged. Its existence in animal tissues, however, suggests that other acids than pyruvic acid may be involved, in which case the production of amines from amino acids may well occur within the animal cell.

CYSTINE AND THE METABOLISM OF SULFUR

Lewis [209] has reviewed the experimental work relating to many problems of sulfur metabolism through the first few months of 1924, and the reader is referred to his excellent paper for a more complete discussion of the subject than can be attempted here.

The metabolism of sulfur in animals relates largely to protein sulfur, since other forms of sulfur do not ordinarily occur in food materials in notable amounts. Of the forms of sulfur occurring in protein, discussion must be largely confined to cystine sulfur, although there are very definite indications that at least one other sulfur compound occurs in the protein molecule. Mueller [210] in 1921 reported the isolation from casein and various other proteins of a sulfur-containing amino acid, to which the empirical formula $C_5H_{11}SNO_2$ was later [211] given. That this compound is a primary cleavage product of proteins is indicated by its ready oxidizability in the animal body [212] and by its influence on the activity of pancreatic amylase, demonstrated by Caldwell [213] in Sherman's laboratory. Its slightly stimulating effect on the saccharogenic activity of this enzyme is similar to the effect of other amino acids obtained from proteins, and in the light of Sherman's theory * as to the cause of this apparent stimulation, is taken to indicate that the Mueller amino acid occurs as such in the protein molecule.

[209] Lewis, H. B., *Physiol. Revs.*, 1924, iv, 394. Reference may also be made to the extensive compilation of Max Kahn and F. G. Goodridge, "Sulfur Metabolism," published in 1926 by Lea and Febiger, Phila.

[210] Mueller, J. H., *Proc. Soc. Exper. Biol. Med.*, 1921-22, xix, 161.

[211] Mueller, J. H., *J. Biol. Chem.*, 1923 lvi, 157. The constitution of the amino acid has been recently determined by Barger and Coyne (*J. Biol. Chem.*, 1928, lxxviii, Proc. p. iii as α-amino-γ-methylthiol-η-butyric acid, and the name methionine has been given to it.

[212] Mueller, J. H., *J. Biol. Chem.*, 1923, lviii, 373.

[213] Caldwell, M. L., *J. Biol. Chem.*, 1924, lix, 661.

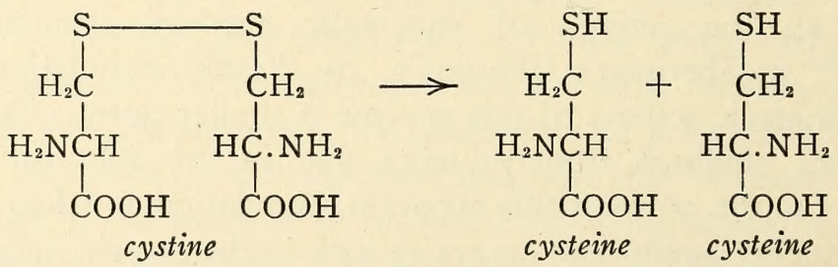
* It is Sherman's theory that this amino acid effect is really due to the inhibition of the hydrolytic destruction of the enzyme, known to be of a protein nature, brought about by the presence of one of the products of its hydrolysis.

The fate of sulfur-containing prosthetic groups in conjugated proteins (such as glucothionic acid and chondroitin sulfuric acid) in metabolism has not been determined, although it is almost certain that the sulfur in these compounds, already completely oxidized, does not undergo further change except in regard to the other chemical groups with which it may be conjugated before elimination.

Thus, the question of sulfur metabolism virtually resolves itself into a study of the metabolism of cystine. It is a matter of great interest and significance that the mercapto grouping found in cysteine, to which cystine appears to be readily reduced in the body, is the only sulfur group that is readily oxidized in animal metabolism. Quoting Lewis on this point, and omitting the literature citations that will be found in his review; "The sulfur of organic sulfides (-C-S-C-) is oxidized with difficulty, if at all. Bivalent sulfur replacing bivalent oxygen as in thiourea, or the thiopyrimidines, is not attacked. . . . The partially oxidized sulfur of sulfonic acids and sulfones is resistant to further oxidation in the organism. * . . . To summarize, it appears that of the various types of sulfur linkages investigated, only mercapto groups or such others as may be hydrolyzed (thiourethane) or reduced (cystine) to form this group are readily oxidized in the organism. An exception to the above statement must be made, however, in the case of mercapto groups attached directly to the benzene nucleus, as in thiophenol or thiocresol, which resist oxidation."

The Oxidation of Cystine

From these considerations, it would appear probable that the first step in the catabolism of cystine in the body would be its reduction to cysteine, a reaction readily brought about *in vitro:*



That this reaction may be brought about *in vivo* is clearly indicated by a number of experimental findings. In 1879, Baumann and Preusse [214] and Jaffé [215] observed that the feeding of monohalogen benzene deriva-

* However, thiosulfates appear to be rather completely (80 per cent) oxidized to sulfates (Nyiri, W., *Biochem. Z.*, 1923, cxli, 160) when administered *per os. See also* Schmidt and Clark, *J. Biol. Chem.*, 1922, liii, 193.

[214] Baumann, E., and Preusse, C., *Ber. chem. Ges.*, 1879, xii, 806.

[215] Jaffé, M., *Ber. chem. Ges.*, 1879, xii, 1092.

tives to dogs induced a synthesis of acetylated cysteine conjugates of the benzene halides (mercapturic acids), which were excreted in the urine. Later investigators have amply confirmed this result, and have shown that on protein-free diets this synthesis does not occur,[216] unless cystine is injected subcutaneously at the same time.[217]

The fact that practically all tissues give a positive color reaction (violet) with sodium nitroprusside ($Na_2Fe(CN)_5.NO$) and ammonia—a test that seems to be specific for the sulfhydryl (mercapto) group—was first noted by Heffter [218] and later by Arnold.[219] Since the tests were also given by protein-free tissue extracts, Arnold [220] suggested that the nitroprusside reaction of tissues was due to free cysteine. Direct proof of the widespread occurrence of cysteine in tissues was afforded by Hopkins [221] who, in 1921, reported the isolation of a dipeptide of cysteine and glutamic acid from tissues, to which he gave the name "glutathione." The importance of glutathione in tissue oxidations will be considered somewhat later in this discussion.

The occurrence of taurine, aminoethylsulfonic acid, in bile as a conjugated taurocholic acid, and in fish and invertebrate muscle (notably in the abolone),[222] together with its close chemical relationship to cysteine, from which it may be prepared *in vitro,*[223] is but another indication of the normal formation of cysteine from cystine in the animal organism. There are other reasons for considering cysteine as the precursor of taurine, which will be given later.

While the three lines of evidence just mentioned point undoubtedly to the possibility that cystine *may* be converted to cysteine in the body, they can hardly be interpreted as indicating that this conversion is an obligate step in cystine catabolism. Evidence of this nature has, however, been obtained by Lewis and McGinty.[224] When the deamination of cystine is prevented by its conjugation with phenylisocyanate, the resulting phenyluraminocystine, when administered to rabbits, is excreted in part in the urine as phenyluraminocysteine. Lewis, Updegraff, and McGinty [225] found that dibenzoylcystine also was excreted in part as dibenzoylcysteine, and that in both cases about 50 per cent of the conjugated compounds appearing in the urine contained cysteine rather than

[216] Thomas, K., and Straczewski, H., *Arch. Physiol.,* 1919, 249.
[217] Kapfhammer, J., *Z. physiol. Chem.,* 1921, cxvi, 302.
[218] Heffter, A., *Med. naturwiss. Arch.,* 1908, i, 81.
[219] Arnold, V., *Z. physiol. Chem.,* 1910-11, lxx, 300.
[220] Arnold, V., *Z. physiol. Chem.,* 1910-11, lxx, 314.
[221] Hopkins, F. G., *Biochem. J.,* 1921, xv, 286.
[222] Kelly, A., *Beitr. chem. Physiol. Path.,* 1904, v, 377; Mendel, L. B., *ibid.,* 1904, v, 582; Okuda, Y., and Sanada, K., *J. Coll. Agr. Imp. Univ. Tokio,* 1919, vii, 77 (*Chem. Abst.,* 1920, xiv, 2806).
[223] Friedmann, E., *Beitr. chem. Physiol. Path.,* 1903, iii, 1.
[224] Lewis, H. B., and McGinty, D. A., *J. Biol. Chem.,* 1922, liii, 349.
[225] Lewis, H. B., Updegraff, H., and McGinty, D. A., *J. Biol. Chem.,* 1924, lix, 59.

cystine. Rose, Shiple, and Sherwin [226] have confirmed these results and have shown that the conversion of cystine to cysteine is a reversible one, since phenyluraminocysteine, injected into rabbits, was in part (to the extent of 14% of the sulfur) converted into the corresponding cystine compound.

The evidence just reviewed appears to justify the conclusion that the first step in the catabolism of cystine is its reduction to cysteine, a conversion from a diamino dicarboxylic acid to a mercapto derivative of alanine. Its next metabolic reaction would presumably be deamination. As a matter of fact, Lewis and Root [227] have shown that when deamination is prevented by conjugating cystine with phenylisocyanate to give phenyluraminocystine, no oxidation of the compound occurs. Rose, Shiple, and Sherwin [226] obtained evidence of some oxidation of phenyluraminocystine, phenyluraminocysteine, and similarly conjugated compounds of cystine and cysteine, even when the substances were injected subcutaneously rather than given *per os*. It is difficult to understand the difference in the results of these two investigations, particularly since the latter ones are not reported fully. In earlier work, Sherwin and Shiple [228] were able to suppress the oxidation *in vivo* of leucine and tyrosine when these amino acids are combined into phenylacetyl derivatives, and Magnus-Levy [229] has reported similar observations with benzoylated amino acids. In any case, the evidence obtained from this type of experimental work may not be as easily interpretable as is currently supposed, since the implied assumption that the blocking of the amino group would have no effect upon the stability of the other groupings cannot be granted. In fact, Brand and Sandberg [230] and others before them have noted that certain combinations of cystine through its amino groups affect markedly the stability of the sulfur, as determined by the ease with which it is removable from the molecule.

A complete inhibition of the oxidation of cystine and cysteine was obtained by Rose, Shiple, and Sherwin only when both the amino and the sulfhydryl group of cysteine were conjugated. Conjugation of the carboxyl group did not appear to affect the oxidizability of these compounds, though the result of the one experiment with cystine hydantoin is of uncertain significance, since the compound was fed rather than injected. As Lewis has pointed out, the interfering influence of the intestinal flora (or the digestive enzymes) in experiments of this nature is an uncertain factor in their interpretation.

[226] Rose, A. R., Shiple, G. J., and Sherwin, C. P., *Amer. J. Physiol.*, 1924, lxix, 518. *See also* Sherwin, Shiple and Rose, *J. Biol. Chem.*, 1927, lxxiii, 607.
[227] Lewis, H. B., and Root, L. E., *J. Biol. Chem.*, 1922, l, 303.
[228] Sherwin, C. P., and Shiple, G. J., *Proc. Amer. Soc. Biol. Chem.*, 1920, p. 26.
[229] Magnus-Levy, A., *Biochem. Z.*, 1907, vi, 555.
[230] Brand, E., and Sandberg, M., *J. Biol. Chem.*, 1926, lxx, 381.

There are several metabolic transformations of cystine in animal metabolism that are peculiar to this amino acid and that connect it with definite organs or definite functions.

Cystine and Liver Metabolism

Among the characteristic constituents of bile are the bile salts, which are sodium salts of a variety of acids. The bile acids distributed the most widely among animals may be grouped into two divisions, the glycocholic acid group and the taurocholic acid group. The glycocholic acids are readily hydrolyzed into glycocoll and nitrogen-free acids known as cholic acids. They are not found, except possibly in traces, in the bile of carnivora or in fish bile. The taurocholic acids are hydrolyzed to give taurine and a cholic acid. They possess the empirical constitution $C_{26}H_{45}NSO_7$. Hammarsten [231] has reported a third group of bile acids in the shark, called scymnol-sulfuric acids; on boiling with hydrochloric acid, these compounds yield sulfuric acid and a non-nitrogenous substance, scymnol ($C_{27}H_{46}O_5$), giving the characteristic color reactions of the cholic acids.

The origin of the bile acids has not been definitely determined and contradictory statements on this point may be found in the literature. Whether they are formed in the liver and there only, or whether, like the bile pigments, they are mainly produced elsewhere than in the liver, which simply excretes them into the bile, cannot be said with any degree of certainty. Foster, Hooper, and Whipple [232] observed that the bile salt excretion of dogs with an Eck fistula * was less than half that of a normal dog under like conditions in all other respects. This finding was interpreted as "direct evidence (of which there has been little available) that the bile acids are formed essentially by liver cell activity." In somewhat analogous experiments, Smyth and Whipple [233] found that chloroform in small doses may almost completely suppress the bile salt output of the liver, and that phosphorus in large doses exerts a distinct depressant effect. These two substances are specific poisons for the hepatic epithelium and the results obtained with them are considered as further direct evidence "that the bile salts are produced by the liver cell and not elsewhere in the body."

It must, however, be remembered that the liver is the main excretory organ for bile salts. Hence, injury to the liver cell would conceivably

[231] Hammarsten, O., *Z. physiol. Chem.*, 1897-8, xxiv, 322.

[232] Foster, M. G., Hooper, C. W., and Whipple, G. H., *J. Biol. Chem.*, 1919, xxxviii, 393.

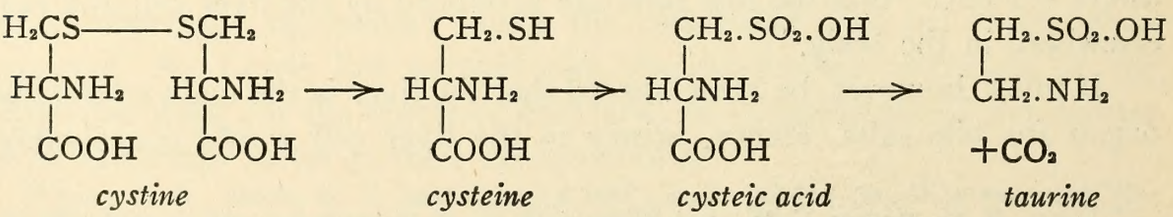
* The Eck fistula effects a direct connection between the portal vein and the posterior vena cava and limits the blood supply of the liver to that coming by way of the hepatic artery. Under these conditions the liver tissue undergoes a certain amount of degenerative change.

[233] Smyth, F. S., and Whipple, G. H., *J. Biol. Chem.*, 1924, lix, 637.

depress the output of bile salts in the bile either (*a*) by depressing the secretion of bile salts from the blood, or (*b*) by depressing the synthetic production of bile salts in the liver cells themselves. Therefore, the mere decrease in output of bile salts following liver injury furnishes no information at all as to the site of formation of these compounds. In the Eck fistula dog of Foster, Hooper and Whipple, the excretion of bile pigments was depressed as greatly as that of bile salts, although only an insignificant fraction of the bile pigment production occurs in the liver.[234] A marked reduction in the output of bile pigments in the Eck fistula dog has also been noted by Whipple and Hooper.[235] Similarly, after chloroform administration to dogs, Smyth and Whipple reported marked reductions in bile pigment output coincident with the reductions in bile salt output, and, although in the phosphorus experiments the parallelism was not so pronounced, it is still evident. Hence these experiments do not constitute a demonstration that the liver cells produce the bile salts they excrete.

A final decision of this important question may be expected from experiments on the total extirpation of the liver by the method of Mann.[236] Up to the present time, the results of the numerous experiments on hepatectomized dogs by Mann and his associates are inconclusive with reference to the relationship of the liver to the formation of bile salts. According to Mann:[237] "Present chemical methods are not sufficiently delicate to determine the accumulation of bile salts in the blood following the obstruction of the common bile duct during the period the hepatectomized animal is kept alive. It would therefore seem impossible to hope to obtain results in the hepatectomized animal until a more delicate method of detection and estimation of bile salts in the blood has been elaborated. We have, however, obtained positive Pettenkofer's tests for bile acids in dialysates of plasma obtained several hours after the liver was removed in dogs whose dialyzed plasma gave no tests for bile acids before operation." Such as it is, therefore, the work at the Mayo Clinic indicates an extra-hepatic production of bile salts.

The synthesis of taurine from cystine is supposed to proceed according to the following scheme—



[234] Mann, F. C., Sheard, C., and Bollmann, J. L., *Amer. J. Physiol.*, 1926, lxxviii, 384.
[235] Whipple, G. H., and Hooper, C. W., *Amer. J. Physiol.*, 1917, xliii, 544.
[236] Mann, F. C., *Amer. J. Med. Sci.*, 1921, clxi, 37.
[237] Mann, F. C., *J. Amer. Med. Assoc.*, 1925, II, 1472.

This scheme involves the action of a decarboxylase in the final reaction, a transformation similar to that of histidine to histamine. However, no experimental support for this mode of formation of taurine is at hand, and, as Lewis points out, it is not inconceivable that, in the formation of taurocholic acid, cystine (or cysteine) may combine with cholic acid before the oxidation of the sulfur group, the conjugated product being then oxidized to taurocholic acid.

While the chemical reactions involved in the formation of taurocholic acid from cystine are thus obscure, the evidence that cystine is one of the precursors of this bile acid appears convincing, though circumstantial in character. It was shown by von Bergmann [238] in 1904 that there was a marked increase in the output of alcohol-soluble sulfur in the bile when cystine and sodium cholate were fed together to a dog. No such effect was induced by cystine alone, though sodium cholate alone did produce a distinct, though less marked, increase in bile sulfur. The experiment of von Bergmann was repeated by Foster, Hooper, and Whipple,[239] using chemical methods that were capable of determining taurocholic acid directly. The results obtained were essentially similar to those of von Bergmann except that the increased output of taurocholic acid induced by cystine administered intravenously and cholic acid given by mouth was not clearly to be distinguished from the increase induced in other experiments [240] under similar conditions by cholic acid alone. The evidence from this line of experimentation seems therefore to be inconclusive, and to require repetition on fasting dogs or dogs subsisting on nitrogen-free rations, since under such conditions only a minimal production of taurocholic acid follows upon the administration of cholic acid alone.

Gibson [241] approached the problem of the relation between cystine and taurocholic acid by the use of a different method, based upon the observation of Jaffé and of Baumann and his pupils that cysteine is conjugated in dogs with bromo- or chloro-benzene to give mercapturic acids, which are eliminated in the urine. If bromobenzene be administered, therefore, to an animal possessing this detoxication mechanism, the resulting artificial cystinuria should deflect the cystine that would otherwise be transformed into taurine and thus depress the production of taurocholic acid. Cats were used in this work and it was in fact found that the subcutaneous injection of bromobenzene decreased the output of alcohol-soluble sulfur in the bile to less than half of the normal. These

[238] von Bergmann, G., *Beitr. chem. Physiol. Path.*, 1904, iv, 192.
[239] Foster, M. G., Hooper, C. W., and Whipple, G. H., *J. Biol. Chem.*, 1919, xxxviii, 421.
[240] Foster, M. G., Hooper, C. W., and Whipple, G. H., *J. Biol. Chem.*, 1919, xxxviii, 379.
[241] Gibson, R. B., *J. Biol. Chem.*, 1909, vi. p. xvi.

results were confirmed by Blum [242] on dogs with biliary fistulas. The evidence that the taurine of taurocholic acid originates from cystine appears to rest largely upon these experiments of Gibson, the details of which have never been published, and of Blum. However, in view of the fact that no other sulfur-containing dietary constituent than cystine—consumed in any considerable amounts—is known to undergo extensive transformation in the body, and in view of the close chemical relations between cystine and taurine and of the convertibility of one into the other *in vitro,*[243] it may be inferred *a priori* that taurocholic acid must originate from dietary cystine.

The possibility of a reverse change of taurine to cystine has recently occupied the attention of several investigators. Mitchell,[244] in experiments upon mice, found that a low casein diet, known to be deficient in cystine, is apparently as effectively improved in nutritive value by taurine as by cystine itself. However, Lewis and Lewis,[245] Beard,[246] and Rose and Huddlestun [247] were unable to obtain evidence indicating that taurine can replace cystine in the diet. In all of these experiments the food intakes of the experimental animals (rats and mice) were uncontrolled, and as a consequence, the results obtained are difficult to interpret, since there is no assurance that the groups of rats compared with respect to rates of growth received equal or comparable amounts of food.* It seems inherently improbable that taurine can replace cystine in the diet, because of its proven stability in metabolism,[248] but nevertheless the possibility suggested by Mitchell's work cannot be disproved by the negative evidence obtained in this type of feeding experiment. The work of Sherwin and his associates [249] on bromobenzene poisoning in dogs, in which it was shown that taurine could not furnish cysteine for this detoxication, does not afford evidence of the impossibility of a conversion of taurine into cysteine in animal metabolism. That such an experimental finding does not disprove the possibility of this metabolic transformation is indicated by the fact that a dog on a nitrogen-free diet does not use cysteine to detoxicate bromobenzene,[249] † although on the

[242] Blum, L., *La semaine med.*, 1906, xxvi, 553. *See* [209].
[243] Friedmann, E., *Beitr. chem. Physiol. Path.*, 1903, iii, 1.
[244] Mitchell, M. L., *Australian J. Exp. Biol. and Med. Sci.*, 1924, i, 5.
[245] Lewis, G. T., and Lewis, H. B., *J. Biol. Chem.*, 1926, lxix, 589.
[246] Beard, H. H., *Amer. J. Physiol.*, 1926, lxxv, 658.
[247] Rose, W. C., and Huddlestun, B. T., *J. Biol. Chem.*, 1926, lxix, 599.

* However, in a private communication, H. B. Lewis has cited food records obtained in the experiments of Lewis and Lewis proving that on the same or smaller amounts of food per 100 grams body weight, the cystine rats grew much faster than the taurine rats. It is unfortunate that these food records do not appear in the published report.

[248] Schmidt, C. L. A., von Adelung, E., and Watson, T., *J. Biol. Chem.*, 1918, xxxiii, 501; Schmidt and Allen, E. G., *ibid.*, 1920, xlii, 55; Schmidt and Clark, G. W., *ibid.*, 1922, liii, 193.
[249] Muldoon, J. A., Shiple, G. J., and Sherwin, C. P., *J. Biol. Chem.*, 1924, lix, 675.

† in these experiments on a dog, as well as in later experiments by the same investigators on a pig,[254] the addition of bromobenzene to a nitrogen-free diet induced marked increases in the excretion of neutral sulfur in the urine, suggesting conjugation with some unoxidized sulfur compound, even though a mercapturic acid could not be identified in the urine.

same type of diet it is continually producing taurocholic acid,[232] the taurine from which presumably originates in cysteine. The most direct interpretation of the first result is that cysteine is not produced in the endogenous catabolism of the tissues; the second result, however, proves that taurine, and presumably cysteine, is continually being produced in endogenous tissue catabolism.

Aside from the continuous production of taurocholic acid on protein-free diets, dogs respond with a marked increase in the output of this bile acid upon the ingestion of protein, indicating the existence of exogenous as well as endogenous factors in the synthesis of taurocholic acid. Foster, Hooper, and Whipple [232] have found that meat protein is particularly effective, and Smyth and Whipple [250] obtained a similar increase with salmon muscle, though gelatin, presumably because of its lack of cystine, did not, in these experiments, cause an increased output of taurocholic acid. Commercial beef extract was also ineffective.

However, it would appear that the effect of dietary protein upon taurocholic acid excretion is not entirely referable to the contained cystine, since cystine alone, or taurine alone, is without effect, although when fed with cholic acid an increased production of taurocholic acid is indicated.[239, 240] Apparently, dietary protein will increase the output of bile acid only when it contains cystine and a precursor of cholic acid. Neither cholesterol, terpene hydrate, camphor or red blood corpuscles appear to give rise to cholic acid in metabolism.[239]

Although taurocholic acid is produced when the nitrogenous metabolism is reduced to the minimum endogenous level by subsistence on carbohydrate and fat diets, the ingestion of substances, such as thyroxin or phlorhizin, which are known to increase tissue catabolism, exert no appreciable influence on the rate of formation of taurocholic acid.[251]

The Sulfur of the Urine

In studies of the metabolism of sulfur compounds, the urinary sulfur is ordinarily divided into three parts, the inorganic sulfate sulfur, precipitable directly by $BaCl_2$, the ethereal sulfate sulfur, precipitable by $BaCl_2$ only after acid hydrolysis, and the neutral sulfur, obtained indirectly by subtraction of the sum of the free and conjugated sulfate sulfur from the total sulfur.

Folin [252] has shown that the inorganic sulfate sulfur in the urine varies closely with the urea nitrogen and is evidently derived mainly from the exogenous protein metabolism.

[250] Smyth, F. S., and Whipple, G. H., *J. Biol. Chem.*, 1924, lix, 647.
[251] Smyth, F. S., and Whipple, G. H., *J. Biol. Chem.*, 1924, lix, 637, 655.
[252] Folin, O., *Amer. J. Physiol.*, 1905, xiii, 66.

The origin of the ethereal sulfate sulfur is not so completely understood, though it is commonly supposed to represent organic sulfates of products originating in the intestinal tract from bacterial action, of the phenol and indole type. These conjugated sulfates are considered as products of a detoxication mechanism probably located in the liver. However, various investigators have suggested an endogenous origin also of these conjugated urinary sulfates, though the continued excretion of ethereal sulfates on nitrogen-free rations cannot be considered as evidence supporting such a suggestion, since bacterial action upon nitrogenous material in the large intestine is still occurring on such rations. In this connection, attention may be called to the recent discovery of Neuberg and Simon [253] of an enzyme, sulfatase, in the liver, kidney, brain and muscle tissue capable of liberating sulfuric acid slowly from conjugated organic sulfates (phenylsulfuric acid). Its significance in the metabolism of sulfur compounds cannot be assessed at present.

The origin of the sulfuric acid occurring in the urinary ethereal sulfates is not definitely known. There is some evidence that the conjugated compound is not formed directly from its two components. For example, Shiple, Muldoon, and Sherwin [254] observed that the feeding of phenol to a pig subsisting upon a nitrogen-free diet increased the output of ethereal sulfates in the urine, and that the feeding of phenol and of cystine simultaneously, increased the ethereal sulfate excretion still more. However, the simultaneous feeding of phenol and sodium sulfate produced no more ethereal sulfate than phenol alone. A similar result was obtained with *p*-chlorophenol and bromobenzene, except that with the simultaneous feeding of cystine, a decrease rather than an increase in the excretion of ethereal sulfates occurred, accompanied by a rise in the neutral sulfur. According to the authors of this report:

> Apparently there are two ways of detoxicating phenolic substances; the one, by combining the poison with a sulfate radical, which is obtained by tissue destruction; the other, by utilizing exogenous cystine, forming eventually a mercapturic acid. This mercapturic acid may then be excreted as such, thereby adding to the neutral sulfur fraction and lessening that of ethereal sulfates, or it may be oxidized to a sulfate and increase the output of ethereal sulfates. This latter case was obtained when cystine and phenol were fed simultaneously.
>
> It is believed that when ethereal sulfates are formed from endogenous sulfur, the toxic substance is joined to some intermediary product of the metabolism of tissue cystine, which product is not formed in the regular oxidation of exogenous cystine. This conjugate is then oxidized to a sulfate.
>
> Our theory is confirmed by the fact that the mercapturic acids (*i.e.*, acetylated cysteine derivatives) of phenol, *p*-chlorophenol, and bromobenzene, are subjected to oxidation by the organism to the extent of 58, 43 and 23 per cent, respectively, in 24 hours.

[253] Neuberg, C., and Simon, E., *Biochem. Z.*, 1925, clvi, 365.
[254] Shiple, G. J., Muldoon, J. A., and Sherwin, C. P., *J. Biol. Chem.*, 1924, lx, 59.

These results of Sherwin and associates are in partial agreement only with the results of very similar experiments on rabbits reported by Rhode [255] after Sherwin's work had been completed. In Rhode's experiments sodium sulfate and sodium thiosulfate did not appear to have any influence on the output of ethereal sulfates in the urine. However, taurine caused a moderate increase and sodium sulfite a marked increase; the increase induced by cystine was about 33 per cent. The feeding of bromobenzene and bromophenol brought about a rise in the output of ethereal sulfates, which was depressed largely by the simultaneous feeding of cystine.

The effect observed in these experiments of the ingestion of sodium sulfite is notable and contrary to the ideas of Sherwin and associates on the mode of formation of ethereal sulfates. Hele [256] has also reported evidence contradictory to the view that the sulfate radical when used in the detoxication of phenolic substances is always of endogenous origin. This investigator reports that sodium sulfate, sodium bisulfite, and cystine are excreted as ethereal sulfates to about *an equal degree* (60 to 80%) when administered to a dog together with the relatively nontoxic guaiacol (carbonate).

Assuming that mercapturic acids are intermediate compounds in the detoxication of certain foreign aromatic substances, which are excreted to some extent as such by the dog (and apparently by the cat also according to Gibson [241]), but which are completely oxidized to ethereal sulfates by most animals, it becomes of interest to inquire concerning the nature of the compounds that are detoxicated in this fashion. Callow and Hele [257] obtained results suggesting that *o*- and *m*-dichlorobenzene are converted to mercapturic acid by the dog, while toluene and *o*-chlorotoluene had no effect upon sulfur output. Coombs and Hele [258] found that *p*-chlorophenol did not give rise to a mercapturic acid and is therefore probably not an intermediary between chlorobenzene and its mercapturic acid. In these latter experiments, phenol and *o*- and *m*-dichlorophenol gave no evidence of mercapturic acid formation.

Although the mechanism of ethereal sulfate formation is thus apparently more involved than that suggested by Shiple, Muldoon, and Sherwin, it is evidently a function directed toward the detoxication of certain types of poisonous compounds normally produced within the intestinal tract of animals by bacterial action. However, the excretion of ethereal sulfates cannot be taken as a safe guide to the extent of putrefaction

[255] Rhode, H., *Z. physiol. Chem.*, 1922-23, cxxiv, 15.
[256] Hele, T. S., *Biochem. J.*, 1924, xviii, 110.
[257] Callow, E. H., and Hele, T. S., *Biochem. J.*, 1926, xx, 598.
[258] Coombs, H. I., and Hele, T. S., *Biochem. J.*, 1926, xx, 606.

occurring there, since it will evidently vary also with the amino acid make-up of the dietary protein, particularly that fraction of it which traverses the small intestine and thus comes under the influence of an active bacterial growth. Furthermore, according to Hanke and Koessler,[259] the type of bacterial flora and the reaction of the medium also influence the production of phenols. Thus, the colon group of organisms forms phenols from tyrosine in alkaline media, but tyramine in acid media.

The neutral sulfur in the urine is determined indirectly and includes all the sulfur groups in the urine except the sulfate group. The constancy in the excretion of neutral sulfur under natural dietary conditions, and its independence from fluctuations in exogenous protein metabolism was first noted by Folin, who assigned to this excretion the same significance as that given to the creatinine excretion. It appears that both excretions, under certain conditions, may be considered as entirely endogenous in origin.

The nature of the sulfur compounds contributing to the neutral sulfur in the urine, aside from an inconsiderable excretion of cystine itself, is not definitely known, though much speculation has been concerned with it, and contradictory experimental results have been obtained. For a review of these investigations the reader is referred to the paper of Lewis.[209]

Many experimental investigations have been concerned with the ratio of sulfur to nitrogen in the urine in starvation and under different dietary conditions, with the hope of throwing light upon the relative lability of the sulfur and nitrogen complexes of protein, the nature of the material stored in the body during protein feeding periods, the relative avidity with which sulfur and nitrogen are retained after starvation, the relative sparing effect of carbohydrate on body constituents containing these two elements, and other questions. A conflicting mass of data has been accumulated, a consideration of which will be found in Cathcart's monograph,[260] Lewis' more recent review,[207] and the recent contributions of Wilson [261] and of Fay and Mendel.[262]

The study of the ratio of sulfur to nitrogen in the urine does not seem to have contributed any definite ideas or information to the science of physiology. Certainly the great importance attached to this type of research by von Wendt [263] has not been justified either by his own work

[259] Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1924, lix, 835, 867.
[260] Cathcart, E. P., "The Physiology of Protein Metabolism," New York and London, 1921, pp. 105 and 123.
[261] Wilson, H. E. C., *Biochem. J.*, 1925, xix, 322.
[262] Fay, M., and Mendel, L. B., *Amer. J. Physiol.*, 1926, lxxv, 308.
[263] von Wendt, G., *Skand. Arch. Physiol.*, 1905, xvii, 211.

or the work of later investigators. The definite interpretation of any ratio between correlated variates is a difficult matter, if not an impossibility. In this particular case the ratio may be affected by the type of protein fed, the relative digestibility of its nitrogen and sulfur, its percentage utilization in anabolism as determined especially by the level of protein fed with reference to the protein requirements, the species of animal used, and the age and nutritive condition of the particular experimental subject, and other factors that might be mentioned. It is conceivable that the liver activity of the subject, by diverting sulfur from urine to feces with no proportionate diversion of nitrogen, might be a potent factor in determining the ratio of sulfur to nitrogen in the urine. From the work of Foster, Hooper, and Whipple it is evident that different foods differ widely in their cholagogue activity.

In the presence of so many disturbing factors to an unequivocal interpretation of the ratio of urinary nitrogen to urinary sulfur, it is not surprising that a study of this ratio has been so barren of result, nor is it surprising that the ratio has been frequently misinterpreted. For example, an increase in the ratio N : S (even during starvation) has been said to indicate a "retention" of sulfur, when as a matter of fact the ratio can give no accurate information as to the relative content of these two elements in the material stored in the body. Only a study of the nitrogen and sulfur balances can answer questions of this character. The urinary ratio seems to be quite generally thought of as being inversely correlated with the retention ratio, bearing a close reciprocal relation with it, although it seems clear that a variable fecal ratio of N : S, or a variable percentage retention of the ingested N and S may affect considerably the urinary ratio without affecting the retention ratio.

It would seem, therefore, that many of the problems it is desired to solve by a study of the urinary ratio of N : S could be much more effectively investigated by a study of the *balances* of these elements in the body.

Cystinuria

From a considerable number of reports in the literature, which have been reviewed by Rosenfeld [264] up to 1920, it appears that in rare cases the metabolism of cystine may be profoundly deranged without any appreciable disturbance in the metabolism of the other amino acids; in some cystinurics a disturbance in the metabolism of arginine and lysine is also indicated by the appearance of the diamines, putrescine and

[264] Rosenfeld, G., *Ergebn. Physiol.*, 1920, xviii, 118.

cadaverine in the urine. This specific metabolic disturbance is indicated by the appearance of abnormally large amounts of cystine in the urine, particularly after protein feeding, and occasionally, though not always, by the formation of urinary calculi containing cystine. For recent reports of observations on cystinurics, papers by Smillie,[265] Flood,[266] Looney, Berglund, and Graves,[267] and Magnus-Levy [268] may be consulted. Lignac has reported [269] a peculiar disturbance of the cystine metabolism of children, characterized *post mortem* by extensive cystine deposits in all parts of the body, but particularly in the kidneys.

A peculiar feature of the metabolism in cystinuria is that free cystine is utilized very completely, only a small fraction being excreted unchanged, although the cystine combined in the protein molecule may be excreted to a considerable extent in the urine. This finding has been interpreted to mean that protein cystine is absorbed as a peptide or in some other form than free cystine. This suggestion, however, requires other support before it can be taken seriously. Another possibility is that cystine may occur in different isomeric forms, as Gortner and Hoffman [270] believe, and that these different isomers may behave differently in metabolism.

The Sulfur Compounds of the Blood

Denis and Reed [271] have written an excellent review of the experimental work concerned with the sulfur compounds occurring in the

Table 28. *Non-Protein Sulfur Compounds in the Blood of Various Animals, from the Analyses of Denis and Reed.*

	Mgms. of S per 100 cc. of Blood					Per Cent on Basis of Total Sulfur			
Source of Blood	Inorganic Sulfates	Total Sulfates	Ethereal Sulfates	Total Sulfur	Neutral Sulfur	Inorganic Sulfates	Total Sulfates	Ethereal Sulfates	Neutral Sulfur
Human ..	1.12	2.01	0.88	4.51	2.50	24.8	44.4	19.6	55.5
" ..	0.35	1.89	1.54	3.75	2.21	9.3	50.6	41.1	59.0
Dog	3.64	8.29	4.65	11.87	3.58	30.6	69.8	39.1	30.1
"	2.02	6.61	4.59	10.08	3.47	20.0	65.5	45.5	34.1
"	3.08	6.16	3.08	8.74	2.58	35.2	69.4	35.2	30.6
Beef	2.35	5.62	3.27	7.29	1.67	32.2	77.0	42.2	22.8
"	2.58	6.18	3.6	8.59	2.51	29.5	71.8	41.9	28.1
Goat	11.28	16.88	5.6	23.96	7.08	47.2	70.7	23.4	29.6
"	6.48	7.16	0.67	12.85	5.70	50.7	55.9	5.2	44.5
Rabbit ...	0.53	2.54	2.01	7.50	4.96	7.1	33.9	2.7	66.1

[265] Smillie, W. G., *Arch. Intern. Med.*, 1915, xvi, 503.
[266] Flood, H. C., *Atlantic Med. J.*, 1923, xxvii, 141.
[267] Looney, J. M., Berglund, H., and Graves, R. C., *J. Biol. Chem.*, 1923, lvii, 515.
[268] Magnus-Levy, A., *Biochem. Z.*, 1925, clvi, 150.
[269] Lignac, G. O. E., *Deut. Arch. klin. Med.*, 1924, cxlv, 139.
[270] Gortner, R. A., and Hoffman, W. F., *J. Biol. Chem.*, 1927, lxxii, 433.
[271] Denis, W., and Reed, L., *J. Biol. Chem.*, 1926, lxxi, 191.

blood. Up to the time of the publication of this article, no definite evidence appears to have been produced as to the forms of sulfur occurring in blood, with the exception of inorganic sulfates. By the use of nephelometric micro methods for the quantitative determination of inorganic and total sulfates and total non-protein sulfur in blood, the following results were obtained by Denis and Reed. The "ethereal sulfates" were determined as the difference between the total non-protein sulfur and the total sulfate sulfur.*

It is interesting to compare the values for the distribution of non-protein sulfur in human blood with average values for the distribution of sulfur in the urine. Folin [272] gives the following averages for urine resulting from a high-protein and a low-protein diet:

Forms of Sulfur in Urine	High Protein Diet	Low Protein Diet
	Per cent	Per cent
Inorganic sulfates	90.0	60.5
Ethereal sulfates	5.2	13.2
Neutral sulfates	4.8	26.3

It is evident that blood ordinarily contains a greater percentage of neutral sulfur than does urine and also considerably more of the conjugated sulfates. It is interesting to note, however, that the distribution of non-protein sulfur in urine more nearly approaches that in blood when the protein metabolism approaches the endogenous level. In pigs maintained on protein-free rations, the neutral sulfur of the urine may make up from 35 to 50 per cent of the total.[254]

While nothing appears to be known about the nature of the conjugated sulfates in blood, some of the unoxidized sulfur compounds have recently been identified. The occurrence of cystine in blood is to be inferred from the fact that amino acids are transported as such from the intestinal tract to the tissues. Its definite isolation from blood has been reported by Abderhalden.[273]

Arnold [220] has reported that protein-free blood-corpuscle filtrates give a positive test with sodium nitroprusside and ammonia, indicating the presence of cysteine. However, Tunnicliffe [274] was unable to find glutathione, diglutaminylcysteine, in rat, rabbit, and human blood, although its occurrence in other tissues was quite general. This finding was in agreement with the earlier report of Hopkins [275] that glutathione

* Reed and Denis have pursued this investigation further in a later publication (*J. Biol. Chem.*, 1927, lxxiii, 623), in which the distribution of inorganic, ethereal, and neutral sulfur between blood corpuscles and plasma is considered.

[272] Folin, O., *Amer. J. Physiol.*, 1905, xiii, 117.
[273] Abderhalden, E., *Z. physiol. Chem.*, 1921, cxiv, 250.
[274] Tunnicliffe, H. E., *Biochem. J.*, 1925, xix, 194.
[275] Hopkins, F. G., *Biochem. J.*, 1921, xv, 286.

was absent from blood plasma. Shortly after the appearance of Tunnicliffe's work, Holden [276] reported the isolation of crystalline glutathione from sheep erythrocytes in an amount equivalent to 5 mgms. per 100 cc. of blood. Using Folin and Looney's colorimetric method for the determination of cystine,[277] Harding and Cary [278] obtained evidence of the occurrence of glutathione in blood. Assuming the total color to be due to the dipeptide, they estimated the glutathione content of ox blood to be approximately 10 mgms. per 100 cc. The investigation of Harding and Cary was primarily only one phase of a general study of milk secretion. They discovered that in dry cows the glutathione content of blood as thus determined was the same whether drawn from the jugular or the mammary vein. With cows in milk there appeared to be a reduction of about 25 per cent in the glutathione content of mammary venous blood. Uyei [279] and Thompson and Voegtlin [280] have analyzed the blood of various species of animals, using modifications of Tunnicliffe's method, and have invariably found evidence of its presence in blood corpuscles and its absence from blood serum. The average analyses of the latter investigators are given in Table 29.

TABLE 29. *The Glutathione Content of the Blood of Different Species of Animals (Thompson and Voegtlin).*

Mgms. per 100 grams sample.

	Defibrinated Blood		Cells		Serum	
	SH-Glutathione	Total Glutathione	SH-Glutathione	Total Glutathione	SH-Glutathione	Total Glutathione
Dog	20	24	55	79	0	0
Rat	22	30	46	53	0	0
Hog	31	39	70	93	0	0
Beef	35	39	73	81	0	0
Calf	36	38	66	74	0	0
Sheep	38	41	86	100	0	0
Rabbit	49	54	104	114	0	0
Guinea pig	49	58	143	151	0	0

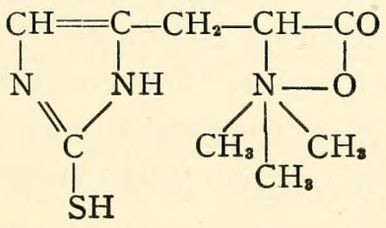
Finally, Hunter and Eagles [281] have isolated from pig blood corpuscles a sulfur-containing product, which on hydrolysis yielded glutamic acid and cystine and which appeared to be chemically identical with a similar product prepared from liver, but which differed in composition from the glutathione of Hopkins. Basing their estimates on the results of the nitroprusside color reaction, Hunter and Eagles estimate that

[276] Holden, H. F., *Biochem. J.*, 1925, xix, 727.
[277] Folin, O., and Looney, J. M., *J. Biol. Chem.*, 1922, li, 427.
[278] Harding, T. S., and Cary, C. A., *Proc. Soc. Exp. Biol. Med.*, 1926, xxiii, 319.
[279] Uyei, N., *J. Inf. Dis.*, 1926, xxxix, 73.
[280] Thompson, J. W., and Voegtlin, C., *J. Biol. Chem.*, 1926, lxx, 793
[281] Hunter, G., and Eagles, B. A., *J. Biol. Chem.*, 1927, lxxii, 133.

blood corpuscles (pig, sheep, and cat) contain 100 to 120 mgms. of glutathione per 100 cc.; since blood plasma contains none of the peptide, the value for whole blood would be approximately half of this figure, quite comparable with the value obtained for the gastrocnemius muscle of a cat, *i.e.*, 47 mgms. per 100 grams.

Thus, the evidence appears to be satisfactory that blood contains glutathione, which appears to be confined to the corpuscles. It is clear, however, in view of the great discrepancy among the estimates of its quantitative occurrence in blood, that more work must be done with improved analytical methods before a definite statement on this point can be made.

The existence of another sulfur-containing constituent in blood has been indicated by recent experimental reports of Hunter [282] and Benedict [283] and their associates. A notable property of this compound is that its sulfur is remarkably stable towards boiling alkali, but is easily removed on oxidation with ferric chloride. The compound obtained at Toronto was called "substance X" by Bulmer, Eagles, and Hunter, and later "sympectothion," [284] because of its firmly bound sulfur. The compound obtained at New York was called "thiasine" by Benedict, Newton and Behre, and has more recently been renamed "thioneine" by Newton, Benedict and Dakin.[285] In a report [286] dated December 17, 1926, the latter investigators identified thiasine with ergothioneine, a base isolated from ergot in 1909 by Tanret and chemically identified as the betaine of thiolhistidine by Barger and Ewins [287] two years later. It thus possesses the following structure:



Its empirical formula is thus $C_9H_{15}N_3SO_2$.

Four days after the publication of this interesting discovery, Eagles and Johnson presented a paper to the editors of the Journal of the American Chemical Society, which was later published,[288] in which this identification of the stable sulfur-containing compound of blood was

[282] Bulmer, F. M. R., Eagles, B. A., and Hunter, G., *J. Biol. Chem.*, 1925, lxiii, 17; Hunter, G., and Eagles, B. A., *ibid.*, 1925, lxv, 623.
[283] Benedict, S. R., *J. Biol. Chem.*, 1925, lxiv, 215; Benedict, S. R., Newton, E. B., and and Behre, J. A., *ibid.*, 1926, lxvii, 267.
[284] Hunter, G., and Eagles, B. A., *J. Biol. Chem.*, 1927, lxxii, 123.
[285] Newton, E. B., Benedict, S. R., and Dakin, H. D., *J. Biol. Chem.*, 1927, lxxii, 367.
[286] Newton, E. R., Benedict, S. R., and Dakin, H. D., *Science*, 1926, lxiv, 602.
[287] Barger, G., and Ewins, A. J., *Tr. Chem. Soc.*, 1911, xcix, 2336.
[288] Eagles, B. A., and Johnson, T. B., *J. Amer. Chem. Soc.*, 1927, xlix, 575.

confirmed. It was also shown in the same report that the "substance X" of Hunter and the "thiasine" of Benedict were identical.

The suggestion that, in view of its evident wide distribution among mammals, the name ergothioneine be contracted to thionene seems a reasonable one. According to Barger [289] the physiological activity of the base is slight and it does not make any significant contribution to the action of ergot.

The thionene of blood, like the glutathione, is evidently confined to the blood corpuscles, in which it occurs in considerable amounts. Newton, Benedict and Dakin have found 14.5 mgms. per 100 cc. of pig blood, although from human blood only 5 mgms. per 100 cc. were obtained. Its physiological significance and in particular its relation to the metabolism of histidine, are problems of great interest and importance.

The Action of Blood on Sulfides

Hydrogen sulfide is a constant product of the action of the bacteria residing in the large intestine of animals on proteins and the sulfur-containing protein derivatives contained in the residues of digestion. It may also be produced by the bacterial reduction in fecal material of inorganic sulfates, according to Kochmann.[290] It may be expected, therefore, that hydrogen sulfide is constantly present in the large intestine and such appears to be the case. Lehmann, [291] in his analyses of intestinal gases, found 1 per cent of hydrogen sulfide, while Wells [292] has reported the occurrence of 66 mgms. of the gas per 100 grams of colon contents. Furthermore, H_2S is readily absorbed by the mucous membrane of the intestine. According to Bunsen,[293] it is absorbed 100 times as rapidly as oxygen, while Peyron [294] observed that when H_2S is injected into *any* of the body cavities it is almost at once detectable in the expired air. The absorption of H_2S by the lungs from the respired air is also rapid.

Hydrogen sulfide is an intensely toxic gas,[295] comparable to cyanogen in this respect. However, its toxicity is not due to a combination of the gas with hemoglobin, as is frequently stated, but to its direct action on the tissues. Hence, the body must possess an effective mechanism for detoxicating the gas, since it is so constantly exposed to its production in the

[289] Barger, G., "The Simpler Natural Bases," London, 1914, p. 47. *See* Tainter, M. L., *Proc. Soc. Exp. Biol. Med.*, 1927, xxiv, 621.
[290] Kochmann, R., *Biochem. J.*, 1920, cxii, 255.
[291] Lehmann, C. G., "Physiological Chemistry," London, 1853, ii, 131.
[292] Wells, H. G., "Chemical Pathology," Philadelphia, 1920, 590.
[293] *Cited from* von Bunge, G., "Textbook of Physiological and Pathological Chemistry," tr. by F. A. Starling, 2nd Engl. ed., 1902, p. 278.
[294] Peyron, J., *Compt. rend. Soc. Biol.*, 1885, xxxvii, 556; 1886, xxxviii, 67, 515.
[295] Mitchell, C. W., and Davenport, S. J., *Pub. Health Rept., U. S. P. H.*, 1924, xxxix, 1.

gastro-intestinal tract itself. Haggard [296] has shown that blood plasma possesses the property of oxidizing H_2S rapidly by the withdrawal of oxygen from the blood corpuscles, and that the products of oxidation, though undetermined, combined in part with the sodium of the plasma. "The rate of oxidation of H_2S in the blood is such that in a comparatively short time many times the lethal amount of sodium sulfide may be administered intravenously to animals without any apparent effect. This explains the comparatively slight toxic properties exhibited by the absorption of sulfides from the intestinal tract," according to Haggard. The sulfide there formed is absorbed slowly and is completely detoxicated in the blood by oxidation. "Large amounts of sulfur may be thus altered and eliminated without the development of any marked physiological effects, although, following the ingestion of sulfur, the breath may be foul with H_2S. In this connection it may be mentioned that about 10 per cent of the sulfur taken by mouth is absorbed as sulfide and later eliminated through the urine.[297] There are, however, a few reported cases of toxic symptoms following the still prevalent administration of sulfur." [298]

The rapid disposal of H_2S by the blood accounts for the fact that the gas exerts no cumulative effect, either when absorbed from the blood or when inhaled; it also explains the rapid recovery in air from non-fatal inhalational poisoning by the gas. Haggard exposed a dog to air containing 5 parts of H_2S per 10,000 for a period of 11 hours and noted no general symptoms, although when the concentration was doubled, death resulted in 15 minutes. Furthermore, it was observed that an animal removed from an atmosphere of H_2S in a state of deep coma recovered its normal appearance and behavior after the lapse of only a few minutes.

While Haggard's work indicated definitely that H_2S gaining access to the blood is rapidly oxidized, no definite evidence was obtained concerning the nature of the products formed. The later investigations of Denis and Reed [299] have supplied this information. Using the same nephelometric methods that were developed in the course of their earlier work on the distribution of the non-protein sulfur in blood, they detected, by analysis of the blood of dogs that had received sodium sulfide by intravenous injection, or by injection into the intestine, increases in the content of inorganic sulfates; no increases in the neutral sulfur fraction were observed. These increases were noted particularly in the

[296] Haggard, H. W., *J. Biol. Chem.*, 1921, xlix, 519.
[297] Cushny, A. R., "Textbook of Pharmacology," Philadelphia, 5th ed., 1913.
[298] Kobert, R., "Lehrbuch der Intoxikationen." Stuttgart, 2nd ed., 1906, i.
[299] Denis, W., and Reed, L., *J. Biol. Chem.*, 1927, lxxii, 385.

case of animals in which the kidneys had been tied off to prevent loss of sulfate in the urine. A much more rapid absorption of sulfide was noted from the duodenum than from the large intestine. Although the increases in blood sulfate were not marked unless the kidneys were incapacitated, the results support the conclusion of Haggard that H_2S is detoxicated by oxidation to H_2SO_4.

Cystine and the Growth of Hair, Wool, and Feathers

The keratins of which hair, wool and feathers are so largely composed are distinguished mainly from the proteins of other tissues in the body by their high content of sulfur and of cystine. A somewhat natural inference from this fact is that the growth of these epidermal structures would be favorably influenced by the consumption of materials rich in cystine and particularly of the keratins themselves. N. Zuntz,[300] following this line of reasoning, has reported the results of feeding hydrolyzed horn preparations to human subjects and to sheep with strikingly favorable effects on hair and wool growth, respectively; on the basis of this work, commercial products of this nature were put on the market under the names of "humagsolan," for human use, and "ovagsolan," to be fed to sheep. Loewy [301] and Blaschko [302] report favorable effects of these horn preparations, the latter work involving pathological cases of defective hair formation or obstinate baldness of seborrheal origin. However, Fuhs [303] repeated Blaschko's studies with "humagsolan" in what appears to have been a more quantitative and critical fashion and found the use of the product by patients suffering from a number of diseases of the scalp was not attended by any appreciable benefit.

It appears, therefore, that the growth-stimulating value of these hydrolyzed keratin preparations on the keratinized tissue in the body has not been satisfactorily demonstrated. Furthermore, the reasoning upon which the expectancy of a positive favorable effect is based is not compelling, since it tacitly assumes either that, under conditions of practical nutrition, hair growth is limited by nutritive factors, and in particular by a deficiency of cystine, or that a prevailing growth of hair (or wool) may be stimulated by increasing the supply of suitable nutritive material. It is somewhat analogous, therefore, to the old superstition that since the brain is rich in phosphorus, phosphorus-rich foods should stimulate brain growth and development.

There are, however, suggestions in the literature that proteins may

[300] Zuntz, N., *Deut. med. Wochnschr.*, 1920, xlvi, 145.
[301] Loewy, A., *Allg. med. Zentral-Z.*, 1920, No. 26; *see Lancet*, 1920, II, cxcix, 1285.
[302] Blaschko, A., *Deut. med. Wochnschr.*, 1920, xlvi, 512.
[303] Fuhs, H., *Wiener klin. Wochnschr.*, 1920, xxxiii, 707.

modify hair growth. Boas [304] reported that a ration containing a commercial dried egg-white preparation as the sole source of protein induced a loss of hair in rats, although freshly prepared egg-white did not have this effect.[305] Hartwell [306] has also published experimental observations leading her to believe that the quality and quantity of the protein fed to rats influence the growth and the loss of fur. However, the cystine content of proteins was not a determining factor in her observations, since gelatin and casein were found to be good correctives.

An interesting and suggestive observation on molting hens has recently been reported by Ackerson, Blish and Mussehl.[307] In an extensive series of studies on the endogenous nitrogen output of hens, they obtained an average figure of 144 mgms. per day per kgm. of body weight for non-molting hens. Molting hens, however, gave much higher values, averaging 219 mgms. per day per kg. body weight. Furthermore, during the molting period, in which a new feather coat is being rapidly formed, the excretion of endogenous nitrogen on a nitrogen-free diet increased in general from the initial to the final stages. In the belief that this increase in endogenous catabolism during molting was due to an increased breaking down of tissue protein to provide cystine for the synthesis of keratin, Ackerson and Blish [308] determined the nitrogen output of molting hens on a diet containing no nitrogen except that contained in a daily quantity of 150 mgms. of cystine. On such a diet, the endogenous nitrogen of a group of 6 molting hens averaged 137 mgms. per day per kg. of weight, as compared with an average value of 239 mgms. for 6 other molting hens receiving no cystine. The individual results obtained in the two groups of birds are quite distinctly different. Evidently in the molting period the nutritive importance of cystine in keratin synthesis is greatly exaggerated.

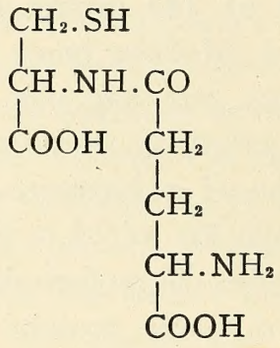
Before leaving the subject of hair growth, it may be pointed out that more success has attended attempts to associate it with endocrine activity, especially the activity of the thyroid,[309] than with dietary factors.

Glutathione

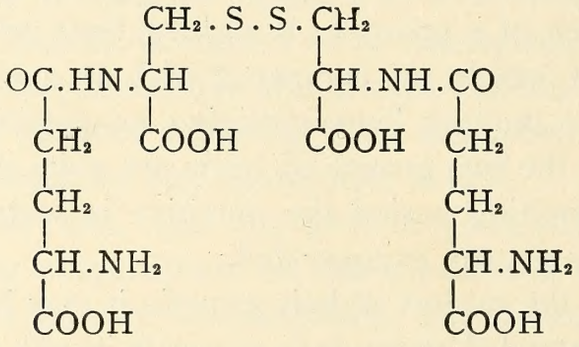
In 1921 Hopkins [310] isolated from yeast, muscle, and mammalian liver a substance which appeared to be responsible for the nitroprusside reaction obtainable from nearly all animal tissues. This reaction had

[304] Boas, M. A., *Biochem. J.*, 1924, xviii, 422.
[305] Boas, M. A., *Biochem. J.*, 1924, xviii, 1322.
[306] Hartwell, G. A., *Biochem. J.*, 1925, xix, 75.
[307] Ackerson, C. W., Blish, M. J., and Mussehl, F. E., *Poultry Sci.*, 1926, v, 153.
[308] Ackerson, C. W., and Blish, M. J., *Poultry Sci.*, 1926, v, 162.
[309] Simpson, S., *Quart. J. Exp. Physiol.*, 1924, xiv, 185; Chang, H. C., *Amer. J. Physiol.*, 1926, lxxvii, 562; Asher, L., and Furuya, K., *Biochem. Z.*, 1924, cxlvii, 425.
[310] Hopkins, F. G., *Biochem. J.*, 1921, xv, 286.

been considered as proof of the presence of compounds containing a sulfhydryl grouping, and the compound obtained by Hopkins in its reduced condition was found to contain cysteine. On hydrolysis, the compound gave glutamic acid as well as cysteine, and its elementary composition corresponded closely with that of a dipeptide of these two amino acids. The compound was called "glutathione" by Hopkins. Of the three possible dipeptides, Quastel, Stewart, and Tunnicliffe [311] found that glutathione in its reduced state was glutaminyl-cysteine, and that the carboxyl group of the dibasic acid involved in the linkage was the one farthest removed from the amino group. Its constitution, therefore, may be represented as follows:



Upon mild oxidation, two molecules of the dipeptide readily combine to form diglutaminyl-cystine:



and upon this equilibrium reaction, according to Hopkins, the biological significance of the compound appears to depend.

The constitution of glutathione appeared to be settled finally when, two years later, Stewart and Tunnicliffe [312] synthesized diglutaminyl cystine and found it to be identical with the natural product in its qualitative reactions, and to be closely equal to it in composition and optical activity. The melting point of the synthesized product was identical with that of the natural product and was not lowered by admixture with it. Recently, however, Hunter and Eagles [313] have attempted to prepare

[311] Quastel, J. H., Stewart, C. B., and Tunnicliffe, H. E., *Biochem. J.*, 1923, xvii, 586.
[312] Stewart, C. B., and Tunnicliffe, H. E., *Biochem. J.*, 1925, xix, 207.
[313] Hunter, G., and Eagles, B. A., *J. Biol. Chem.*, 1927, lxxii, 147.

glutathione from animal tissues according to the method of Hopkins, but have obtained products differing in composition from the product of Hopkins, particularly in respect to its sulfur content which was much lower than that of diglutaminyl cystine. On the basis of these results they are inclined to question the conclusion that glutathione has the constitution ordinarily assigned to it. Their analyses suggest to them that it may be a tripeptide containing serine, as well as glutamic acid and cysteine. However, as Hopkins [314] points out, the partial desulfurization of glutathione during its preparation is not an uncommon eventuality. This fact, in conjunction with the discordant sulfur contents of the different glutathione preparations of Hunter and Eagles, detracts considerably from the force of their contention; it seems a fair judgment, therefore, that no serious objection exists to the constitution of glutathione as originally announced.

The importance of glutathione in cellular metabolism resides in the ease with which it may be changed *in vivo* from the sulfhydryl (SH) to the disulfide (S.S) form. It can be both reduced and oxidized under the influence of factors shown to be present in the tissues themselves. Its capacity as a hydrogen acceptor and a hydrogen donator was demonstrated by Hopkins and Dixon,[315] using methylene blue as a reduction indicator. When minced fresh muscle tissue is washed until it no longer reduces methylene blue and is suspended in a buffer solution, the addition of glutathione to the solution, either in the reduced or oxidized form, will restore its power to reduce methylene blue. A condition is thus established which under anaërobic conditions continuously reduces methylene blue until an equilibrium is reached, by a transport of hydrogen from tissue to dye through the reversible changes induced in the added glutathione. It was further shown that molecular oxygen can serve as the hydrogen acceptor (as well as methylene blue), since washed tissue suspensions that, by themselves take up but little oxygen, absorb many times more in the presence of glutathione (400 c. mm. per gram of dry tissue). After such treatment it will have wholly lost its power to reduce methylene blue in the presence of glutathione.

It is of particular interest that these oxidation-reduction activities that are established when glutathione is brought into relation with a tissue which has been simply well washed with cold water show little if any quantitative difference if the same tissue has been first *heated to 100°C.* or has been completely extracted with *boiling* water. Furthermore, they are scarcely affected if the tissue has been subsequently dried

[314] Hopkins, F. G., *J. Biol. Chem.*, 1927, lxxii, 185. *See also* Johnson, J. M., and Voegtlin, C., *ibid.*, 1927, lxxv, 703.

[315] Hopkins, F. G., and Dixon, M., *J. Biol. Chem.*, 1922, liv, 527.

and powdered. "The facts suggest," according to Hopkins and Dixon, "that coexisting in living tissues with the specialized enzymic mechanisms is a thermostable mechanism for oxidations and reductions. Materials in some close association with structural elements are oxidized, anaërobically or aërobically, with the coagency of the sulfur group in glutathione."

In a later publication, Hopkins [316] has presented experimental data indicating that the direct source of transportable hydrogen in muscle tissue is the fixed SH in the tissue proteins. These experiments revealed an oxidation of certain pure proteins by glutathione, in either the sulfhydryl or disulfide state, along peculiar lines:

> The oxidation proceeds only when and while the protein itself displays (as shown by the nitroprusside reaction) an SH group. The proteins of water-extracted muscle contain this group, and suffer the oxidation in question in neutral or faintly alkaline solutions. In acid systems (pH 3.0-4.5) no oxidation of protein occurs.
>
> In this oxidation the protein SH is oxidized and the nitroprusside reaction disappears, while the total oxygen taken up amounts to ten times the oxygen equivalent of the SH. The protein can then be again reduced; the SH group reappears and (with glutathione) a further uptake of oxygen becomes possible, which is again greatly in excess of the oxygen equivalent of the SH. Successive reductions and oxidations of this kind can be carried out, till finally the protein takes up at least 10 cc. per g. and possibly much more.
>
> At any stage the oxidized protein (showing no nitroprusside reaction) can be reduced by contact (as a solid phase) with strong solutions of glutathione (SH), cysteine, or thioglycollic acid, so that it again displays an intense nitroprusside reaction. Such a "reduced" protein then again takes up oxygen on the lines already described. When insoluble protein is in contact with solutions of glutathione, or other thiol compounds, equilibria are established between sulphur groupings in the solid (protein) phase and sulphur groups in solution. Mutual oxidation and reduction occur as reversible processes.
>
> Whilst the proteins of water-extracted muscle undergo oxidation and reduction, on the lines described, in their native state, certain other proteins (those of blood serum, for example) need to be first denaturated. In an insoluble form the proteins of serum can be so reduced by contact with strong solutions of thiol compounds as to display an intense nitroprusside reaction. They then behave like the muscle proteins.

Thus, the phenomena observed with washed tissue preparations by Hopkins and Dixon all appear to be referable to the interaction of tissue proteins, glutathione, and molecular oxygen or methylene blue. Lipoid-free muscle preparations behave in this relation in the same way as the tissue from which the lipoids have not been extracted.

The behavior of glutathione in the presence of aqueous emulsions of unsaturated fatty acids or lecithin can apparently be explained only on the assumption that the former is serving as an oxygen carrier by the formation of an intermediary peroxide. In such emulsions oxidized glutathione is inert, but reduced glutathione induces a vigorous oxidation in acid media, though only a limited one in neutral media. The two types

[316] Hopkins, F. G., *Biochem. J.*, 1925, xix, 787.

of oxidation may be represented by the two following schemes, in which A represents the autoxidator (glutathione) and B an oxygen acceptor (unsaturated fatty acid):

$$(a)\quad AO_2 + 2B \longrightarrow A + 2BO$$
$$(b)\quad AO_2 + B \longrightarrow AO + BO$$

The first scheme represents a catalyzed oxidation, such as occurs in acid systems (pH = 3.0 to 4.5). The second scheme represents an induced or coupled oxidation, such as occurs in neutral or alkaline systems (pH 7.4 to 7.6). Under the latter condition, the SH group of glutathione is itself oxidized irreversibly to S.S, and the unsaturated bonds of the fatty acids are oxidized simultaneously in such a way that an equipartition of oxygen occurs.

Certain important differences were observed by Hopkins between the oxygen uptake of emulsions of unsaturated fatty acids in the presence of glutathione and the oxygen uptake of glycerides of such acids. In particular, the latter system exhibited a "period of induction" lasting 1 to 3 hours, in which the absorption of oxygen was extremely slow.

It is beyond the scope of this chapter to consider the numerous experimental contributions relating to the function and mode of action of glutathione, and the associated theoretical speculations. Reference, however, should be made to the important work of Meyerhof [317] on the activity of cysteine and thioglycollic acid in oxidation-reduction systems, paralleling in many ways the experimental work at Cambridge; to the work of Warburg and Sakuma,[318] showing that the SH group of thiol compounds is not strictly autoxidizable, but is dependent upon the presence of minute traces of iron, a finding that was later confirmed by Harrison [319] for glutathione, at least to the extent that the autoxidation of this thiol form is relatively very slow in the absence of all but irremovable traces of iron; and to the contribution of Kendall and Nord,[320] leading to the conclusion "that an oxygen addition product of glutathione is the *sine qua non* of the activity of glutathione in physiological processes of oxidation in so far as a reversible system is concerned."

While the probability that glutathione will later be shown to have intimate relations with specific metabolic reactions in the body must be admitted, such relations have not been demonstrated at the present time, and the subject of its functions in the body is largely speculative. It is perhaps significant that the activity of glutathione with reference to the

[317] Meyerhof, O., "Chemical Dynamics of Life Phenomena," Philadelphia and London, 1924, Chapter II. *Also Pflüger's Arch. ges. Physiol.*, 1923, cxcix, 531.
[318] Warburg, O., and Sakuma, S., *Pflüger's Arch. ges. Physiol.*, 1923, cc, 203. *Also* Sakuma, *Biochem. Z.*, 1923, cxlii, 68.
[319] Harrison, D. C., *Biochem. J.*, 1924, xviii, 1009.
[320] Kendall, E. C., and Nord, F. F., *J. Biol. Chem.*, 1926, lxix, 295.

oxidation of glucose and glucose derivatives has not yet been demonstrated. On the other hand, the intimate relation of the compound with cellular respiration is indicated by the demonstrated antagonism between cyanides, specific respiratory poisons, and cystine, cysteine, or glutathione. The interesting literature on this subject is discussed by Meyerhof [317] and by Voegtlin, Johnson and Dyer.[321] The latter investigators report additional evidence to support the view that, *in vitro,* cyanides reduce S.S groups and in turn are oxidized from CN to CNO, a group only one thirty-second as toxic as the original cyanide.

The Glutathione Content of Tissues

The first satisfactory quantitative method for the determination of glutathione in tissues, involving extraction with 10 per cent trichloroacetic acid and titration with iodine, using sodium nitroprusside as an outside indicator, was used by Tunnicliffe [274] in Hopkins' laboratory. The method obviously determines the reduced SH glutathione; however, the incubation of tissue before analysis, which was shown to lead to a reduction of S.S glutathione, did not increase the analytical values obtained. The conclusion was therefore drawn that, in the tissues of normal animals, glutathione is present chiefly in the reduced form. It was also found that the glutathione sulfur in tissue extracts accounted for the greater part of the neutral sulfur, thus indicating the presence in only inappreciable amounts of other non-protein organic sulfur-containing compounds in tissues.*

Tunnicliffe reported percentages of glutathione ranging from 0.15 to 0.22 (average 0.18) for yeast; for rat liver, percentages from 0.16 to 0.21 (average 0.18) were obtained; for rabbit liver, 0.22 to 0.35 (average 0.24); for rat muscle, 0.033 to 0.035 (average 0.034), and for rabbit muscle, a mean value of 0.041, with little variation from it. A mean value of 0.17 per cent is reported for kidney of unstated origin. No glutathione was found in whole blood or in fresh hen's egg. Among the analyses reported, the relatively low value for muscle, in which oxidation processes may reach an intensity unparalleled in other tissues, is noteworthy. It may indicate that glutathione is unrelated to carbohydrate oxidation.

Uyei [279] and Thompson and Voegtlin [280] have reported the average

[321] Voegtlin, C., Johnson, J. M., and Dyer, H. A., *J. Pharm. Exp. Therap.*, 1926, xxvii, 467.

* However, the recent discovery of (ergo-) thioneine in blood may necessitate a revision of this conclusion. If this thiolhistidine derivative is present in tissue and is extracted by 10 per cent trichloroacetic acid (and we have found no indication in its described properties that it would not) it would be oxidized by iodine to a compound quite analogous to S.S. glutathione containing two directly linked sulfur atoms. Furthermore, the latter compound has the property of taking up more iodine.[285] Hunter and Eagles,[281] however, have obtained indications of the absence of thioneine from yeast and liver.

analyses for different organs of different animals summarized in Tables 30 and 31.

TABLE 30. *Average Reduced Glutathione Content of the Different Tissues of Rats of Different Body Weight, According to Thompson and Voegtlin.*

Range of Weight	No. of Rats	Percentage of Glutathione on Fresh Basis			
		Liver	Skeletal Muscle	Brain	Kidney
20– 26	108	0.261	0.024	0.102	0.156
41– 59	135	0.171	0.025	0.099	0.111
64– 77	30	0.154	0.023	0.074	0.052
86– 94	33	0.135	0.027	0.078	0.045(?)
142–178	45	0.179	0.032	0.132	0.115
188–206	20	0.177	0.034	0.112	0.094
245–276	30	0.204	0.024	0.040	0.019

In agreement with Tunnicliffe, Uyei found that the reduced glutathione of animal tissue (liver) accounts for practically all of the organic sulfur extracted by trichloroacetic acid. No consistent effect of fasting on the glutathione content of tissues is revealed by the values in Table 31.

TABLE 31. *The Average Reduced Glutathione Content of the Tissues of Fed and Fasted Animals, According to Uyei.*

	Fed Animals				Fasted Animals			
	Rabbit	Rat	Dog	Guinea Pig	Rabbit	Rat	Dog	Guinea Pig
No. of animals averaged	2	2	3	2	3[b]	4[c]	1[d]	3[e]
Organs	%	%	%	%	%	%	%	%
Liver	0.33	0.23	0.21	0.29	0.30	0.24	0.17	0.23
Lung	0.14	0.11	0.08	0.13	0.16	0.08	0.04	0.12
Muscle	0.038	0.033	0.050	0.037	0.050	0.021	0.045	0.043
Kidney	0.15	0.18	0.14	0.19	0.11	0.12	0.14	0.18
Spleen	0.24	0.16	0.09	0.22	0.21	0.11	0.10	0.22
Heart	0.08	0.08	0.08	0.07	0.08	0.06	0.09	0.07
Adrenal	0.16	...	...	0.13	0.17	...	...	0.16
Testicle	...	0.15	0.06	0.14	0.10	...	0.06	0.17
Ovary	0.17	...	...	...	...	...	...	...
Brain	0.09	0.12	0.06	0.10	0.08	0.08	0.06	0.08
Lens	0.33	...	0.27[a]	0.33	0.29	...	0.27	0.31
Pancreas	...	...	...	...	...	...	...	...
Marrow	0.08	...	...	...	0.08	...	...	...

[a] For the pancreas, a value of 0.15 per cent was found for a female dog, and one of 0.08 per cent for a male dog.
[b] Fasted 24, 48 and 72 hours.
[c] Three fasted 3 days and one 5 days.
[d] Fasted 3 days.
[e] Two fasted 2 days and one 7 days.

The values in both of the above investigations indicate a relatively low content of skeletal muscle tissue in glutathione, and a relatively high content in glandular tissue. This relation parallels the resting respiratory

metabolism of these tissues as determined *in situ*. A compilation of some values of this character is given in Table 32.

TABLE 32. *The Oxygen Consumption of Different Tissues and Organs at Rest In Situ.*

Expressed in cc. O_2 per min. per gram of tissue.

Tissue	Oxygen Consumption	Animal	Observer
Skeletal muscle	0.0037	Horse	Chauveau and Kaufman
" "	0.0045	Cat	Verzár
Cardiac muscle	0.010	Dog	Barcroft and Dixon
Submaxillary gland	0.027	Cat	Barcroft and Piper
Liver	0.005–0.018	Cat	Barcroft and Shore
Intestine	0.005–0.009	Dog	Brodie and Vogt
Kidney	0.025–0.060	....	Neumann
Adrenal gland	0.045	Cat	Neumann
Lungs	0.015	Dog	Evans

The most outstanding disparity between the two sets of data relates to the relation between liver and kidney tissue. In glutathione content the liver is distinctly higher, while with reference to the intensity of respiration, the kidney is distinctly more active than the liver, a fact recently confirmed by Rohr.[322]

The glutathione data of Thompson and Voegtlin do not indicate any clear differences for animals of different age, except that for the youngest (smallest) group, the highest values were generally obtained. However, for the entire bodies of rats, they report average glutathione contents of 0.036 per cent for the new born, 0.032 per cent for nurslings, 0.031 per cent for weanlings, and 0.023 per cent for rats over half grown (137 to 170 grams in weight). For rat embryos, a distinctly decreasing glutathione content with increasing age was observed, an experimental finding identical with that reported by Murray [323] for chick embryos.

In an investigation of the glutathione content of malignant transplantable mammalian tumors in rats, Voegtlin and Thompson [324] found them to contain relatively large concentrations, comparable to those of liver, although the necrotic portion was free of glutathione. They also found that with the growth of the tumor the glutathione content of the rest of the body decreases, an observation probably related to cancer cachexia.

It has been stated that both Tunnicliffe and Uyei obtained definite indications that glutathione accounts for practically all of the organic non-protein sulfur of animal tissues. Thompson and Voegtlin [280] also investigated this matter, availing themselves of the recently described

[322] Rohr, K., *Z. physiol. Chem.*, 1923, cxxix, 248.
[323] Murray, H. A., *J. Gen Physiol.*, 1925-26, ix, 621.
[324] Voegtlin, C., and Thompson, J. W., *J. Biol. Chem.*, 1926, lxx, 801.

colorimetric method of Sullivan [325] for the estimation of cystine, cysteine, and glutathione. This method is based upon the color reaction of cysteine with β-naphthoquinone-4-sodium sulfonate. Cystine will also react after reduction (with sodium cyanide), and glutathione after hydrolysis. Through the coöperation of Dr. Sullivan, it was found that the trichloroacetic acid tissue extracts prepared from liver, muscle, brain, and kidney of the rat gave no test for either cysteine or cystine, although the color develops in cysteine concentrations as dilute as 25 parts per million. However, using the same reaction, Sullivan earlier has reported [326] that "in strong polyneuritis on a rice diet, the tissues of pigeons have little if any cysteine, but do contain cystine; while on a rice diet plus vitamin, the tissues of the pigeons contain cysteine as well as cystine. In extreme polyneuritis the reducing capacity of the tissues seems to be suspended."

The presence of cystine in animal tissues has also been reported by Cloetta [327] for ox kidney, by Drechsel [328] for the liver of the dolphin, and by Hunter and Eagles [329] for pig liver. It is suggested by the latter investigators "that the free cystine in liver may be of importance in the formation of taurocholic acid and that one of the manifestations of cystinuria may lie in a hepatic failure to metabolize the free cystine of liver."

TYROSINE AND PHENYLALANINE

A study of the oxidizability in the animal body of aromatic compounds reveals many interesting instances of the specificity of metabolic processes, particularly if attention is confined to the oxidizability of the benzene nucleus itself. Among the many aromatic compounds whose fate in the body has been investigated, only a very few are oxidized completely. Phenyl substituted fatty acids are oxidized only with reference to the aliphatic side chains, the aromatic residue consisting either of benzoic acid or phenylacetic acid, depending upon whether the side chain contained an odd or an even number of carbon atoms.

Among the few aromatic substances that have been found to be oxidized completely in the body are:

α-amino-β-phenyl-propionic acid (phenylalanine) and its corresponding α-hydroxy and α-ketonic acids.

p-hydroxy-phenylalanine (tyrosine) and its corresponding α-hydroxy- and α-ketonic acids.

2-5-dihydroxyphenylacetic acid (homogentisic acid).

p-methylphenylalanine and *p*-methoxyphenylalanine.

[325] Sullivan, M. X., *Pub. Health Rpts., U. S. P. H.*, 1926, xli, 1030.
[326] Sullivan, M. X., *Proc. Amer. Soc. Biol. Chem.*, 1924, vi, 3; *J. Biol. Chem.*, 1925, lxiii, p. xi.
[327] Cloetta, A., *Ann. Chem.*, 1856, xcix, 299.
[328] Drechsel, E., *Z. Biol.*, 1896, xxxiii, 85.
[329] Hunter, G., and Eagles, B. A., *J. Biol. Chem.*, 1927, lxxii, 167.

As illustrations of the slight changes in chemical structure that may determine whether aromatic compounds are or are not completely oxidized in the body, it may be pointed out that if the amino group in phenylalanine, or the hydroxyl or keto groups in its derivatives, is shifted from the alpha to the beta position in the side chain, the oxidation of the benzene nucleus is not possible. Similarly, with tyrosine, if the hydroxyl group is changed from the para to the ortho or the meta positive in the ring, oxidation of the benzene nucleus is also prevented.

The study of the method of oxidation of tyrosine and phenylalanine has been conducted along three general lines, *i.e.*, (*a*) the isolation and identification of derivatives of the amino acids from the urine of normal animals to which they or their suspected metabolites have been administered, (*b*) the fate of suspected metabolites when perfused through a surviving liver, or (*c*) when administered to alcaptonurics.

The latter two methods have given the most suggestive results. Phenylalanine and tyrosine themselves readily lead to the production of acetoacetic acid when perfused through a surviving liver under proper conditions, and when administered to an alcaptonuric they readily lead to the excretion of increased amounts of homogentisic acid (2,5-dihydroxyphenylacetic acid) in the urine. Hence, it appears to be a reasonable inference that all of the normal (obligatory) intermediary metabolites of these amino acids will behave likewise, and, conversely, that derivatives of either amino acid that are neither convertible into acetoacetic acid or homogentisic acid under these conditions are in all probability not normal metabolites of phenylalanine or tyrosine.*

Before proceeding with the consideration of the results obtained from these methods of investigation, a brief description of the metabolic anomaly known as "alcaptonuria" will be given.

Alcaptonuria

Alcaptonuria is a very rare metabolic disease that was first described in 1859 by von Bodeker. Since then, 50 or 60 cases have been observed and reported in the literature. All but one of the cases reported have been in human subjects; the exception was but lately observed by Lewis [330] in a rabbit.

Alcaptonuria is characterized by the production of a urine which assumes a dark brown or black color on standing, which reduces an alkaline solution of copper sulfate in the hot or an ammoniacal solution of silver nitrate in the cold, and which forms an orange-colored precipi-

* This statement should probably be restricted to those metabolites formed previous to acetoacetic acid or to homogentisic acid, since it is possible that metabolism beyond these stages may involve irreversible transformations.

[330] Lewis, J. H., *J. Biol. Chem.*, 1926, lxx, 659.

tate with Millon's reagent. The urine does not react to Nylander's test for sugar (reduction of alkaline bismuth solutions) nor does it rotate the plane of polarized light. Baumann and Wolkow in 1891 were the first to demonstrate that these properties of alcaptonuric urine are due to the presence of homogentisic acid, which can be readily isolated from it in relatively large amounts. The ratio between homogentisic acid and total nitrogen is quite constant in alcaptonuric urine,[331] ranging from 45 to 100 to 55 to 100.

The metabolic disturbance apparently involves only tyrosine and phenylalanine. These amino acids when given to an alcaptonuric may be recovered quantitatively in the urine as homogentisic acid.[332] The disease, however, has no pathological significance, since it does not affect the general health of people afflicted with it. Since children in which this metabolic disturbance exists may grow in an apparently normal way to a perfectly normal adult existence, it is evident that the tyrosine and phenylalanine of the food must be readily available for anabolic syntheses as in normal individuals. Only the oxidation of these aromatic amino acids is abnormal or incomplete. Statements that the disease is hereditary or is of especially high incidence among the children of consanguineous marriages have been frequently made and disputed, so that evidently more data are needed to settle definitely these very interesting questions.

The Oxidation of Phenylalanine and Tyrosine in the Body

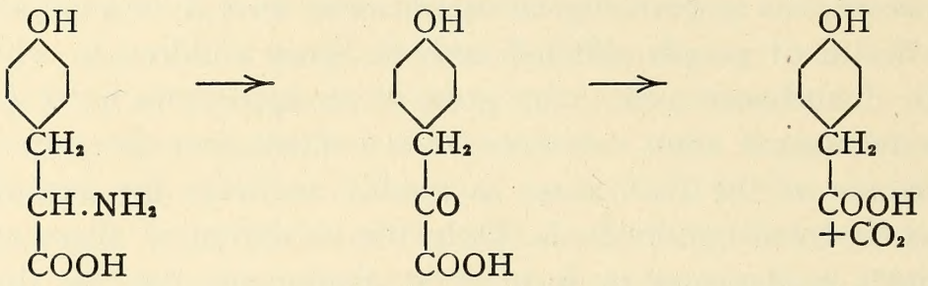
It is a peculiar fact, to which attention has already been directed (see p. 278), that in the case of phenylalanine the most probable primary product of deamination appears to be an α-hydroxy acid, while in the case of tyrosine it appears to be an α-ketonic acid. Phenylpyruvic acid, although totally combustible in the body [333] and convertible into phenylalanine in the liver [334] and into homogentisic acid in the alcaptonuric organism,[335] does not yield acetoacetic acid in the liver;[336] in fact, when perfused with substances that are acetoacetic-acid formers, such as phenylalanine, tyrosine, leucine, p-hydroxyphenylpyruvic acid and n-butyric acid, it depresses or totally inhibits their normal conversion into acetoacetic acid.* Phenyllactic acid, however, conducts itself in all these respects like phenylalanine. On the contrary, p-hydroxyphenylpyruvic acid, but not p-hydroxyphenyllactic acid, yields homogentisic

[331] Ravold, A., and Warren, W. H., *J. Biol. Chem.*, 1910, vii, 465.
[332] Falta, W., *Biochem. Centralblatt*, 1904-05, iii, 175.
[333] Knoop, F., "Der Abbau aromatischer Fettsäure im Tierkörper," Freiburg, 1904.
[334] Embden, G., and Schmitz, E., *Biochem. Z.*, 1910, xxix, 423.
[335] Neubauer, O., and Falta, W., *Z. physiol. Chem.*, 1904, xlii, 81.
[336] Embden, G., and Baldes, K., *Biochem. Z.*, 1913, lv, 301.

* However, curiously, no inhibition of acetoacetic-acid formation from isovalerianic acid or η-caproic acid due to the simultaneous perfusion of phenylpyruvic acid was observed.

acid and acetoacetic acid.[335, 337] From the work of Kotake and Mori and associates,[338] it appears that *l*-*p*-hydroxyphenyllactic acid is formed in the animal organism normally only by the asymmetric reduction of *p*-hydroxyphenylpyruvic acid, similar to the asymmetric reduction of phenylglyoxylic acid to *l*-mandelic acid.[339]

The transformations of the aromatic amino acids from this point on are less clearly demonstrable. Considering tyrosine first, it seems extremely improbable that further oxidation of *p*-hydroxyphenylpyruvic acid occurs in the side chain at this stage, since the expected product would be *p*-hydroxyphenylacetic acid, which is entirely resistant to oxidation in the body:[340]



tyrosine — *p*-hydroxyphenylpyruvic acid — *p*-hydroxyphenylacetic acid

Hence, it is probable that the next step in the catabolism of *p*-hydroxyphenylpyruvic acid is the oxidation or cleavage of the benzene nucleus. The substitution of hydroxyl groups into the benzene ring is suggested by the conversion of tyrosine into homogentisic acid in alcaptonurics. The question arises, therefore, whether this is a step in the normal metabolism of tyrosine beyond which the alcaptonuric organism is unable to go, or whether it is a transformation that occurs only in alcaptonuria and has no particular significance in normal metabolism.

In either case it is important to consider the probable method of conversion of tyrosine into homogentisic acid. An initial difficulty is the fact that in tyrosine (or in *p*-hydroxyphenylpyruvic acid) the hydroxyl group is in position 4 with reference to the side chain, while in homogentisic acid this position is vacant, the two hydroxyl groups being in positions 2 and 5. The removal of the 4-hydroxyl group is inherently improbable, since the reduction of a phenol group in the animal body is difficult if not impossible. A way out of the dilemma was first suggested by Meyer,[341] who called attention to the observations of

[337] Neubauer, O., and Gross, W., *Z. physiol. Chem.*, 1910, lxvii, 219; Schmitz, E., *Biochem. Z.*, 1910, xxviii, 117.

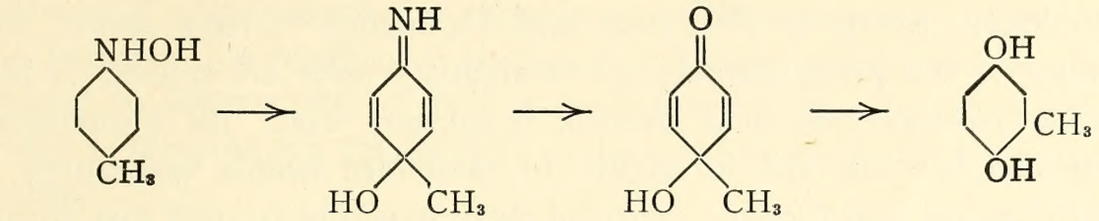
[338] Kotake, Y., and Okagawa, M., *Z. physiol. Chem.*, 1922, cxxii, 201; Mori, Y., and Kanai, T., *ibid.*, 1922, cxxii, 206.

[339] Neubauer, O., *Deut. Arch. klin. Med.*, 1908-09, xcv, 211.

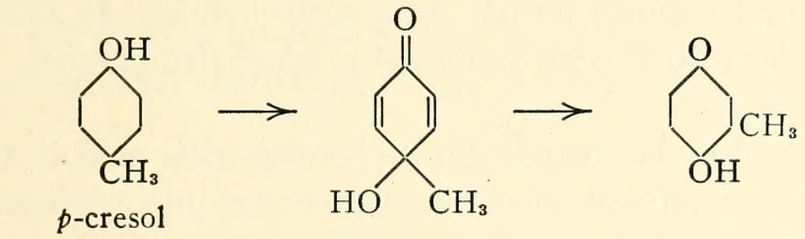
[340] Salkowski, E., and Salkowski, H., *Z. physiol. Chem.*, 1882-83, vii, 171. *Also* Sherwin, C. P., *J. Biol. Chem.*, 1918, xxxvi, 309.

[341] Meyer, E., *Deut. Arch. klin. Med.*, 1901, lxx, 447.

Bamberger [342] that *p*-tolylhydroxylamine may be readily converted into methylhydroquinone by treatment with hot dilute H_2SO_4, and that *p*-cresol, by oxidation with Caro's reagent, can be changed to *p*-toluquinol and methyl-hydroquinone. These reactions involve intramolecular rearrangements similar to that required in the conversion of *p*-hydroxyphenylpyruvic acid to homogentisic acid, and may be written as follows:

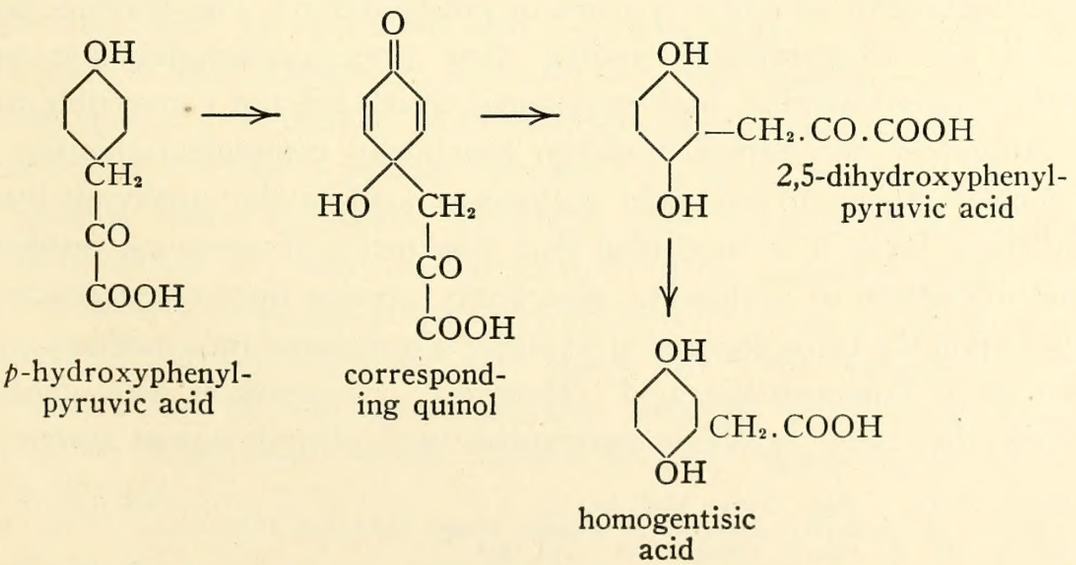


p-tolylhydroxylamine — imido-toluquinone — toluquinone — methyl-hydroquinone



p-cresol

Hence, on the assumption of the intermediate formation of an unstable quinonoid compound, the *p*-hydroxyl group in tyrosine becomes a necessary structure for the transformation into homogentisic acid, rather than an embarrassment. In conformance with this idea, Neubauer [339] has shown that of the three possible mono-hydroxyphenylpyruvic acids, only the para compound is convertible into homogentisic acid in the alcaptonuric. Hence, Meyer, Friedmann and Neubauer [341, 343, 339] picture the formation of homogentisic acid from *p*-hydroxyphenylpyruvic acid as follows:



p-hydroxyphenyl-pyruvic acid — corresponding quinol — homogentisic acid

[342] Bamberger, E., *Ber. chem. Ges.*, 1895, xxviii, 245; *ibid.*, 1903, xxxvi, 2028.
[343] Friedmann, E., *Beitr. chem. Physiol. Path.*, 1908, xi, 304.

Neubauer [339] has shown that 2-5-dihydroxyphenylpyruvic acid is convertible into homogentisic acid.

Of particular significance to this proposed scheme is the finding of Dakin [344] that *p*-methylphenylalanine and *p*-methoxyphenylalanine, from which a quinonoid substance could not be formed, are not converted into homogentisic acid in the alcaptonuric body, but are apparently oxidized completely. Similarly Fromherz and Hermanns [345] have shown that neither *m*- nor *p*-tolylalanine, nor *m*-methyltyrosine are capable of producing homogentisic acid. Hence, it appears that "the alcaptonuric organism has not lost its ability to catabolize simple derivatives of phenylalanine and tyrosine provided their structure is such that formation of substances of the type of homogentisic acid is excluded." However, these experimental findings do not necessarily indicate, as Dakin believes, that homogentisic acid is an abnormal product characteristic of alcaptonuria.

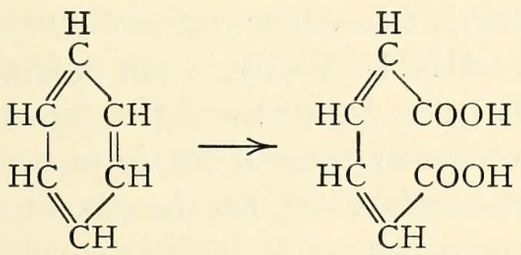
No definite scheme for the breakdown of homogentisic acid in the normal organism has been offered, though its conversion into acetoacetic acid in the surviving liver has been demonstrated by Embden.[346]

The fact that the body is able to oxidize homogentisic acid with moderate ease [346] and that it is an acetoacetic-acid former must be considered as evidence favoring the view that it is a normal metabolite of tyrosine. Also Abderhalden [347] has isolated a small amount of homogentisic acid from the urine of normal men after the ingestion of a large amount of tyrosine. However, Wakeman and Dakin [348] have brought forward evidence that has been interpreted to mean, if not that homogentisic acid is an abnormal metabolic product, at least that it is not an obligate metabolite of either tyrosine or phenylalanine. The evidence consists of the experimental finding that *p*-methylphenylalanine and *p*-methoxyphenylalanine, both substances which are not convertible into para-quinonoid derivatives, undergo practically complete oxidation in the normal organism and yield acetoacetic acid in the surviving liver. From these facts, it is concluded that "the series of reactions resulting in the production of acetoacetic acid from tyrosine does not necessarily depend upon the prior formation of either a quinonoid intermediary substance or of homogentisic acid." However, it appears at least equally probable that these synthetic para-substituted phenylalanines pursue a

[344] Dakin, H. D., *J. Biol. Chem.*, 1911, ix, 151.
[345] Fromherz, K., and Hermanns, L., *Z. physiol. Chem.*, 1914, xci, 194.
[346] Embden, H., *Z. physiol. Chem.*, 1893, xviii, 304.
[347] Abderhalden, E., *Z. physiol. Chem.*, 1912, lxxvii, 454.
[348] Wakeman, A. J., and Dakin, H. D., *J. Biol. Chem.*, 1911, ix, 139.

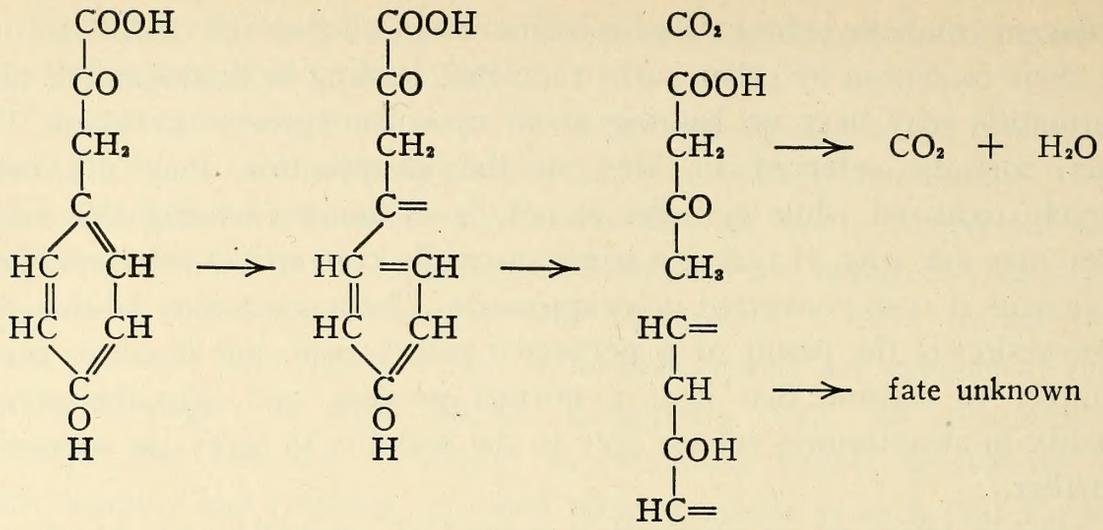
different catabolic course from tyrosine itself, so that the demonstration of their oxidation by other paths than that leading to homogentisic acid formation may have no bearing at all upon the tyrosine problem. The fact, already referred to, that in the alcaptonuric they are completely oxidized while tyrosine is not, is evidence favoring this view. One may ask why, if tyrosine is not normally convertible into homogentisic acid, it is so converted in alcaptonuria. The answer may be that this conversion is the result of a perverted metabolism, but it seems much simpler to assume that it is a normal process, and that the abnormality in alcaptonuria relates only to the inability to carry the oxidation further.

Dakin offers as an alternative method of catabolism of *p*-hydroxyphenylpyruvic acid an immediate opening of the benzene ring, suggested by the finding by Jaffé [349] that small amounts of muconic acid can be isolated from the urine of dogs which had been given benzene—



although Mori,[350] who repeated Jaffé's work, was unable to substantiate his results. In picturing the formation of acetoacetic acid from *p*-hydroxyphenylpyruvic acid without the intermediary formation of homogentisic acid, the possibility presents itself that the carbon atoms adjacent to the phenol group might, in the course of some molecular rearrangement, be converted into β-hydroxybutyric acid and then into acetoacetic acid. However, Dakin rejects this suggestion, since acetoacetic acid can be formed from aromatic amino acids in which the para position in the ring is already occupied by a methyl or methoxy or other grouping. Dakin concludes, therefore, that "tyrosine yields acetoacetic acid in such a way that the carbon atom of the carboxyl group and that in the α-position in acetoacetic acid are derived from the α and β carbon atoms of the phenylalanine side chain, while the remaining β and γ carbon atoms of the acetoacetic acid molecule are derived from two adjacent carbon atoms in the benzene nucleus," according to the following scheme—

[349] Jaffé, M., *Z. physiol. Chem.*, 1909, lxii, 58.
[350] Mori, Y., *J. Biol. Chem.*, 1918, xxxv, 341.



It is possible that the fragment containing the four carbon atoms that occupied the 2, 3, 4, and 5 positions in the benzene nucleus are also converted into acetoacetic acid, though no positive evidence concerning its fate in metabolism, except that it is completely oxidized, is available.

This scheme of Dakin's accounts very well for the fact that phenylserine, $C_6H_5.CHOH.CHNH_2.COOH$, is not oxidized completely in the body, but is converted into benzoic acid,[351] since the β-substitution in phenylserine would obviously prevent the formation of acetoacetic acid as indicated. Dakin also believes [352] that the scheme also accounts for the failure of phenylaminoacetic acid, γ-phenyl-α-aminobutyric acid and their derivatives to undergo complete oxidation in the body; however, phenylacetic acid and γ-phenylbutyric acid and in fact all phenyl-substituted fatty acids of this character are themselves incapable of complete oxidation, for reasons that Dakin's theory of the manner of cleavage of the benzene ring cannot explain, except for those acids yielding benzoic acid by β-oxidation.

Furthermore, Dakin's scheme does not appear to account for the failure of *o*-tyrosine and *m*-tyrosine to be oxidized in the body. Flatow [353] has shown that these compounds when fed to rabbits are excreted as the corresponding acetic acid derivatives. On the other hand, the theory of Neubauer that tyrosine is converted into homogentisic acid in the course of its oxidation is consistent with the failure of these isomers of tyrosine to be broken down in the body, since it is quite conceivable that hydroxyl substitution in the ortho or meta position of the benzene nucleus would interfere with the formation of the necessary intermediate quinonoid compound. Blum [354] has in fact shown that neither *o*-tyrosine

[351] Dakin, H. D., *J. Biol. Chem.*, 1909, vi, 235.
[352] Dakin, H. D., "Oxidations and Reductions in the Animal Body," 2nd ed., p. 90, London and New York.
[353] Flatow, L., *Z. physiol. Chem.*, 1910, lxiv, 367.
[354] Blum, L., *Arch. exptl. Path. Pharm.*, 1908, lix, 269.

nor *m*-tyrosine are convertible into homogentisic acid in the alcaptonuric, while Neubauer [339] has proven the same to be true for their α-ketonic derivatives.

Dakin has proposed his theory of the cleavage of the benzene ring as an alternative to the theory of Neubauer and others that hydroxylation of the ring precedes its cleavage. However, the objections he has raised to the prior formation of homogentisic acid do not appear to be well-founded; he has shown that phenylalanine derivatives whose structure precludes the formation of homogentisic acid may nevertheless undergo complete oxidation in the normal organism. This, however, is not equivalent to proving that tyrosine is not normally oxidized by way of homogentisic acid. In fact, the observation of Dakin that the phenylalanine derivatives with which he worked are not converted into homogentisic acid in the alcaptonuric while tyrosine is, definitely suggests that the former are catabolized differently from the latter in the normal organism also. Neither of the two theories appears to be in contradiction to available experimental observations nor are they mutually exclusive. It is suggested, therefore, that they may both be correct, and that the catabolism of *p*-hydroxyphenylpyruvic acid be considered as involving, first, conversion into homogentisic acid according to the scheme of Neubauer and others, and, second, cleavage of the benzene ring according to the scheme of Dakin. In thus reconciling the two views, it is, perhaps, suggestive that the hydroxyl groups of homogentisic acid are attached to those carbon atoms in the benzene nucleus (2 and 5) at which cleavage of the ring occurs according to Dakin.

The conversion of phenylalanine into phenyllactic acid, rather than phenylpyruvic acid, by deamination, has already been mentioned as the logical interpretation of the results of the experimental methods that have been used in the elucidation of intermediary metabolic reactions. Certainly phenylpyruvic acid does not appear to pursue the same catabolic path as phenylalanine, though it may be produced from it by massive administration of phenylalanine to dogs.[355] The main pathway of catabolism of phenyllactic acid is difficult to understand in view of the following experimental observations:

(*a*) Oxidation to phenylpyruvic acid yields a compound which, unlike phenylalanine itself, is not oxidizable to acetoacetic acid,[336] though it is completely oxidizable in the body by some other pathway.[335] A peculiar situation is revealed by the fact that phenylpyruvic acid is convertible into homogentisic acid,[335] which may in turn yield acetoacetic acid in the

[355] Kotake, Y., Masai, Y. and Mori, Y., *Z. physiol. Chem.*, 1922, cxxii, 195.

liver.[356] Such apparent inconsistencies indicate the difficulty of properly interpreting experimental results of this nature.

(*b*) On the other hand, oxidation in the ring to either ortho-, meta-, or para-hydroxy phenyllactic acid is contraindicated, since these compounds are not convertible into homogentisic acid.[339]

A way out of the dilemma is suggested by the important experiments of Embden and Baldes,[336] who reported that perfusion of the liver with *dl*-phenylalanine leads to the production of small amounts of *l*-tyrosine, while in control experiments with no amino acid additions or with additions of aliphatic amino acids no demonstrable amounts of tyrosine could be isolated. The results suggest that phenylalanine is converted into tyrosine before deamination, a transformation which is also indicated by the isolation of *l*-hydroxyphenylpyruvic acid from the urine of rabbits after phenylalanine administration, in experiments by Kotake, Masai, and Mori.[355] Another possibility would be the deamination of phenylalanine to phenyllactic acid, and the simultaneous oxidation of the latter, in both ring and side chain, to *p*-hydroxyphenylpyruvic acid, the conversion of which to homogentisic acid has been demonstrated.[339] It is to be noted that the hydroxylation of phenylalanine or phenyllactic acid in either the ortho or meta position in the benzene nucleus seems ruled out by the fact that such compounds are resistant to further ring oxidation in the body.

Hence, the metabolism of phenylalanine and of tyrosine appears to be largely the same. It should be noted, however, that while the conversion of phenylalanine to tyrosine in normal metabolism appears extremely probable, the reverse change does not, since the reduction of a phenolic group has never been observed in the animal organism.[352, p. 87] In other words, hydroxylation of the benzene ring, as in the conversion of phenylalanine to tyrosine or to tyrosine derivatives, appears to be an irreversible process. These considerations are of importance to the question of the indispensability of these amino acids in animal nutrition.

Tyrosinase

A tyrosine-oxidizing enzyme, tyrosinase, has been detected in both vegetable and animal tissues. It produces from tyrosine a pink or red pigment, which on further action changes to a brown or black melanin-like substance.

Tyrosinase was first discovered [357] in 1896 by Bertrand in plants, and a constant and excellent source of the oxidase is dialyzed potato

[356] Embden, G., Salomon, H., and Schmidt, F., *Beitr. Chem. Physiol. Path.*, 1906, viii, 129.
[357] Vernon, H., "Intracellular Enzymes," London, 1908, p. 123.

juice, the color changes of which on standing in air have been traced to the tyrosine-tyrosinase reaction. Two years later, Biedermann showed that a similar enzyme is present in the meal worm (*Tenebrio molitor*), and recently Raper and Speakman [358] have presented evidence on the identity of the tyrosinase from these two sources. Its occurrence has also been reported in certain insects (*Lepidoptera*), crustaceans (crayfish), molluscs (cephalopods), sponges, and finally in bacteria.[359] Miss Durham [360] obtained distinct evidences of the presence of tyrosinase in the skin of rats, rabbits, and guinea pigs. Aqueous extracts of such skins, incubated with tyrosine and a trace of ferrous sulfate, darkened and finally deposited a black melanin-like substance. The action was destroyed by boiling the extract, and was greatly accelerated up to temperatures of 37°C. Gortner [361] has suggested that the presence or absence of certain inhibitory substances (phenols) on the tyrosine-tyrosinase reaction may explain the dominant and recessive white plumage and hair coloration which occur in the animal kingdom, and the two forms of white which occur among flowers.

The action of tyrosinase on tyrosine, by which the latter is converted into pigmented substances, has been found to involve—

1. Production of a red pigment.
2. The red substance changes spontaneously to a colorless substance.
3. The colorless substance on exposure to oxygen blackens to form **melanin.**

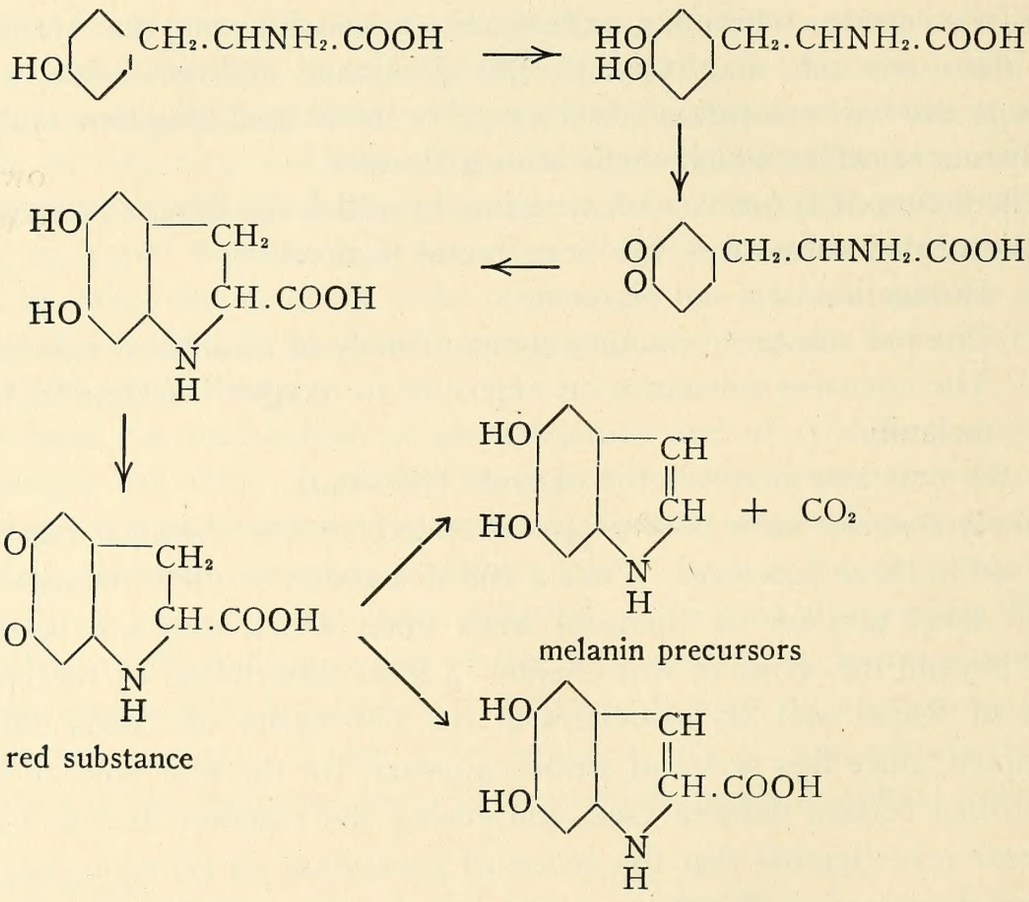
Only the first step involves the enzyme tyrosinase.

Many theories have been proposed to explain the chemical reactions involved in these processes. While a full discussion of these theories and of the large amount of chemical work upon which they are based is quite beyond the scope of this chapter, a brief description of the recent work of Raper and his associates at the University of Leeds will be attempted, since this series of studies appears, for the first time, to have established certain definite facts concerning the reaction. It was found by these investigators that the action of tyrosinase on tyrosine does not involve deamination,[362] contrary to earlier theories, and that the melanin finally formed contains a slightly higher nitrogen content than tyrosine itself, *i.e.*, 8.40 per cent, as compared with 7.73 per cent.[363] Probably the first substance formed from tyrosine is *l*-3,4-dihydroxyphenylalanine,[364]

[358] Raper, H. S., and Speakman, H. B., *Biochem. J.*, 1926, xx, 69.
[359] Stepp, C., *Biochem. Z.*, 1923, cxli, 42.
[360] Durham, F. M., *Proc. Roy. Soc. London*, 1905, lxxiv, 310.
[361] Gortner, R. A., *J. Biol. Chem.*, 1911, x, 113.
[362] Happold, F. C., and Raper, H. S., *Biochem. J.*, 1925, xix, 93.
[363] Raper, H. S., and Wormall, A., *Biochem. J.*, 1925, xix, 84.
[364] Onslow, M. W., and Robinson, M. E., *Biochem. J.*, 1925, xix, 420.

the isolation and identification of which was first reported by Raper.[365] This compound is converted into the red pigment by tyrosinase. Later, Raper [366] identified two compounds produced on the decolorization of the red pigment, apparently by reduction. When the reduction was allowed to proceed spontaneously, 5,6-dihydroxyindole was mainly formed with a simultaneous evolution of CO_2. When the reduction was hastened by the addition of sulfurous acid, decarboxylation did not occur to so great an extent, and the main product formed was 5,6-dihydroxyindole-2-carboxylic acid. These two substances are regarded by Raper as the immediate precursors of the melanin ultimately produced.

The action of tyrosinase on tyrosine, through the formation of the melanin precursors, may therefore be pictured as follows, according to Raper:



The red pigment formed is assumed to be the 5,6-quinone of dihydroindole-2-carboxylic acid. The demonstration of the formation of the indole nucleus from tyrosine by an enzyme (or a mixture of enzymes) having a wide distribution in nature, including mammalian tissue, is of great potential significance.

It is of interest to note that phenylalanine is not attacked by tyro-

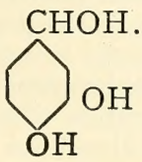
[365] Raper, H. S., *Biochem. J.*, 1926, xx, 735.
[366] Raper, H. S., *Biochem. J.*, 1927, xxi, 89.

sinase,[362] nor is *p*-hydroxyphenylpyruvic acid.[363] The ability of tyrosinase to oxidize other amino acids than tyrosine in the presence of tyrosine or of other phenols has been investigated by McCance [367] and by Happold and Raper.[362]

The occurrence of 3,4-dihydroxyphenylalanine in the pods of the broad bean (*Vicia faba*) was observed by Guggenheim [368] in 1913, and its occurrence in the Georgia velvet bean was reported by Miller [369] in 1920. According to Miller its concentration in the velvet bean is sufficient to cause physiological effects in men, *i.e.*, vomiting and purging.

Adrenalin

A substance of great physiological activity occurring in the medulla of the adrenal (or supra-renal) glands, has been found to possess a structure suggesting its relationship to tyrosine. This substance has received a number of names, such as adrenalin, supra-renin, epinephrin, and adrenine, but will be referred to as adrenalin in this discussion. For an excellent account of its history and its chemical and physiological properties, the reader is referred to the monograph of Barger on "The Simpler Natural Bases." Its isolation from the adrenal glands was accomplished independently by Takamine and Aldrich in 1901, and its constitution established by the researches especially of Pauly, Jowett and von Furth, and Friedmann, and finally its synthesis by Stolz in 1904 and later by others. Adrenalin has been identified as *l*-3,4-dihydroxyphenylethanolmethylamine—



While nothing is known concerning the precursor of adrenalin to the body, the fact that it is a hydroxylated aromatic derivative has favored the belief that it is derived from tyrosine. In fact, Halle [370] in 1906 reported increases in the adrenalin content of the adrenal glands when they were incubated with tyrosine. Ewins and Laidlaw,[371] however, were unable to confirm this observation.

In connection with his latest investigations on the nature of the tyrosine-tyrosinase reaction, Raper [366] makes the following significant remark:

[367] McCance, R. A., *Biochem. J.*, 1925, xix, 1022.
[368] Guggenheim, M., *Z. physiol. Chem.*, 1913 lxxxviii, 276. *See also* Torquati, T., *Arch. farm. sperim.*, 1913, xv, 213, 308; Hirai, K., *Biochem. Z.*, 1921, cxiv, 67; Waser, E., and Lewandowski, M., *Helvet. chim. acta.*, 1921, iv, 657.
[369] Miller, E. R., *J. Biol. Chem.*, 1920, xliv, 481.
[370] Halle, W. L., *Beitr. chem. Physiol. Path.*, 1906, viii, 276.
[371] Ewins, A. J., and Laidlaw, P. P., *J. Physiol.*, 1910, xl, 275.

"The production of 3-4-dihydroxyphenylalanine from tyrosine by a biological method of oxidation overcomes an obstacle which previously existed in accepting the view that tyrosine is the parent substance of adrenalin. The presence of an enzyme in the supra-renal gland which oxidized tyrosine has not yet been demonstrated, but it is probable that 3-4-dihydroxyphenylalanine may be produced somewhere in the body. Bloch [372] and Bloch and Schaaf [373] have presented evidence that it is the only substrate which is effective for the production of melanin in epithelial pigment cells by 'dopa' oxidase. This enzyme does not attack tyrosine, but its mere existence and its apparent specificity render the production of 3-4-dihydroxyphenylalanine in some situation in the body very probable."

A comprehensive statement of the physiological effects and of the function of adrenalin, or epinephrin, in so far as they are understood at present, is given by Abel [374] in the following quotation:

> No other principle of the body has of recent years received such exhaustive study along chemical, physiological and pharmacological lines. It passes in the medical literature under various names, as epinephrine, adrenalin, supra-renin, adrenin, etc. The official designation adopted by the U. S. Pharmacopea is epinephrin, a name first employed by me in my earliest investigations on this subject and derived from ἐπι upon, and νεφρ's the kidney.
>
> It is now well established that this hormone is continuously being given off in almost infinitesimal amounts to the venous blood and thence distributed throughout the body in a very high dilution but one which suffices to give tone to, to relax slightly, or to inhibit the activity of the various tissues of the body. There is still much discussion, however, as to the exact significance of the hormone under normal conditions as well as in circumstances of emergency. It is argued that it is not in itself essential to the continuance of life, since extirpation of the medullary portion, or chromophil tissue, of the suprarenal gland, which, as we have seen, is the source of the hormone, is not followed by death. It must always be borne in mind, however, that it is quite impossible to extirpate every trace of medullary or chromophil tissue, since tissue of this character is found not only in the accessory suprarenals, but also, to a small extent, in every one of the sympathetic ganglia located in the abdominal and thoracic cavities. Suppose, for example, that an investigator has removed both suprarenals and all of the accessible accessory suprarenals, he will still have left behind a quite indeterminate, though admittedly a small, amount of chromophil tissue. Now, it is a well established fact that glandular organs in general, and especially the glands of internal secretion, have a very large factor of safetly, which is only another way of saying that a small remnant of the gland can carry on in emergencies in place of the whole. Under the circumstances I cannot believe that it has been proved with certainty that epinephrine is not essential to the continuance of life. But perhaps this is a question of academic importance only, since all of the more recent work of pharmacologists and physiologists leaves no doubt as to the functional significance of the hormone, be it only in cases of emergency.
>
> Leaving now the question as to how far epinephrine exerts any function in the animal organism, I purpose to give you a brief summary of the extraordinary physiological discoveries that have been made in respect to this product in recent years. Foremost there is a striking parallelism in the action of epinephrine and excitation of the sympathetic nervous system. In a word, epinephrine stimulates

[372] Bloch, B., *Z. physiol. Chem.*, 1917, xcviii, 226.
[373] Bloch, B., and Schaaf, F., *Biochem. Z.*, 1925, clxii, 181.
[374] Abel, J. J., *Bull. Johns Hopkins Hospital*, 1926, xxxviii, 1.

or inhibits the action of practically all structures innervated by the sympathetic system in a way analogous to electrical stimulation of the sympathetic nerves going to that part. To give examples, it accelerates the rate of the heart beat (a sympathetic reaction), entirely comparable to that which follows electric stimulation of the accelerator or sympathetic nerves to the heart. It relaxes the stomach and the intestines. On the contrary, it causes increased tonus of the pyloric, ileocœcal and internal anal sphincter, as does stimulation of the splanchnic nerve. It causes a thick, viscid saliva to be secreted, an increase in lacrymal secretion, an increase in bile secretion, a tremendous increase in the sweat secretion of some animals, as in the horse and sheep (but has no effect on this secretion in most animals), contraction of the erectores pilorum, contraction of both the gravid and non-gravid uterus in many animals, relaxation of the non-gravid uterus, as in the cat, the rat, the mouse and the guinea-pig; contraction of the trigone and relaxation of the fundus of the bladder; relaxation of the bronchi, especially if they previously have been put into a state of spasm, dilatation of the pupil of the eye and exophthalmus, conversion of the glycogen of the liver and muscles into blood sugar, polycythæmia and relaxation of the coronary vessels. All these reactions may be produced by electrical stimulation of the appropriate sympathetic nerve to the part in question.

The point of attack of epinephrine in all of the above instances is not the nerve end itself, but an intermediate anatomical unit, something which appears to be neither pure nerve tissue, nor yet a pure muscle or gland cell, but an unidentified something called the receptive substance, a myoneural or adenoneural junction, as the case may be. It must be evident that we have in epinephrine a substance which must be classed in respect to its action and dosage with our most powerful alkaloids. It is truly speaking, as I have said above, an autogenetic drug. And we are not surprised to learn that it has both antagonistic and synergistic relations not only to others of our autogenetic drugs, or hormones, but to many alkaloids or other drugs as ergotoxin, apocodeine, curare, atropine, physostigmine, histamine and the like. As a drug this hormone is of considerable importance in practical medicine. Its extraordinary ability to cause constriction of small arteries and veins whenever the drug can be applied to them, its power to cause relaxation of spastically contracted bronchioles and its power to resuscitate an arrested heart are to be noted among the facts that account for its widespread use. Naturally, a drug of such extraordinary potency and such a variety of actions must always be used with care and only by those who are endowed with intelligence. We are fortunate indeed that our suprarenal glands do not seem able, even in times of emergency, to throw out into the general circulation any but lower concentrations of this hormone. Life would be incompatible with the sudden liberation into the blood stream at any one moment of a considerable amount of it, say, 15 or 20 mgm. The two suprarenals of an adult contain together at the most only nine milligrams of the principle. After acute infections and various exhausting diseases this amount is very much reduced, but on recovery regeneration of the substance seems to occur within a few days.

Thyroxin

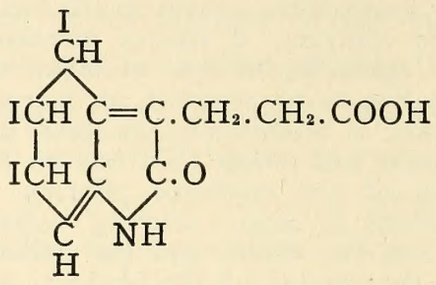
Thyroxin, the active principle of the thyroid gland (or one of the active principles), was first isolated by Kendall [375] in a crystalline form in 1915, by a laborious series of chemical manipulations. It evidently exists in the gland in combination in a protein molecule (Oswald's iodothyreoglobulin), from which it is liberated by mild alkaline hydrolysis. Its most characteristic chemical property is its high content of iodine, 65 per cent according to Kendall.

In later papers, published partly in coöperation with Osterberg,[376]

[375] Kendall, E. C., *J. Amer. Med. Assoc.*, 1915, xliv, 2042.

[376] Kendall, E. C., *J. Biol. Chem.*, 1919, xxxix, 125; Kendall and Osterberg, A. E., *ibid.*, 1919, xl, 265.

the conclusion was drawn from a large mass of chemical data that thyroxin is an indole derivative, and, in 1919, the following formula was assigned to it:



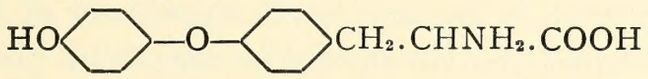
Although the work of Kendall was considered as conclusively establishing the constitution of thyroxin, Harington,[377] in 1926, raised a number of criticisms against this proposed formula. It appeared to Harington that the stability of thyroxin, particularly towards boiling alkali, "is scarcely consistent with the supposed existence in the molecule of a 2-4-6-trihydro-2-4-6-triiodoindole nucleus," and that its relative stability towards oxidizing agents and, when pure, towards acids, "are not such as would be expected from a consideration of the known properties of indole derivatives of a somewhat similar constitution to that proposed for thyroxin." However, in the same year, Hicks [378] reported the results of studies of the absorption spectra of thyroxin, 2-hydroxyindole-3-propionic acid, and tryptophane, and concluded that the absorption curves for these substances are related sufficiently to warrant the conclusion that the indole nucleus is present in thyroxin. Furthermore, the effect of the substitution of iodine in isatin on its absorption spectrum was found to be similar to the difference in the absorption spectra of thyroxin and the uniodized indole compounds.

Nevertheless, Harington continued his investigations inspired by the belief that thyroxin is not an indole derivative. Using a slight modification of Kendall's method of preparing thyroxin from thyroid glands,[379] he was able to prepare an amount corresponding to 0.12 per cent of the dried gland or 0.027 per cent of the fresh gland (Kendall's yield = 0.0011%). About 14 per cent of the total iodine of the glands was thus isolated as thyroxin. However, upon analysis the crystalline product was found to have the empirical composition $C_{15}H_{11}O_4NI_4$, rather than $C_{11}H_{10}O_3NI_3$, the formula proposed by Kendall.

Having prepared 100 grams of crude crystalline thyroxin, its chemical nature was then studied. Desiodo-thyroxin was prepared from the thyroxin by reduction with the palladium-hydroxide-calcium-carbonate catalyst of Busch and Stöve. It was found that an amount of hydrogen

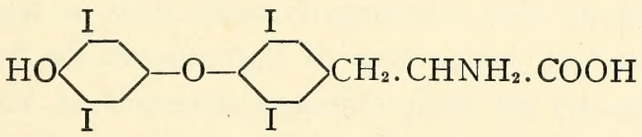
[377] Harington, C. R., *J. Biochem.*, 1925, lxiv, 29.
[378] Hicks, C. S., *J. Chem. Soc.*, 1925, cxxvii, 771.
[379] Harington, C. R., *Biochem. J.*, 1926, xx, 293, 300.

was taken up equivalent to the iodine liberated, *i.e.*, 4 molecules per molecule of thyroxin. The desiodo-tyrosine thus produced had a composition coresponding to the formula $C_{15}H_{15}O_4N$. It gave Millon's reaction, indicating the presence of a phenol group, and the ninhydrin reaction for an alpha-amino group. The whole of its nitrogen was liberated by HNO_2, and it formed salts with both acids and bases. The proportion of C and H suggested the presence of two benzene rings in the molecule, and this suggestion was supported by direct experimental evidence. Concerning the kind of linkage of the two benzene rings, the evidence suggested an oxide linkage, and on the basis of this suggestion a compound was synthesized which proved to be identical with the desiodo-thyroxin prepared from thyroid material. This compound was β-[4(4′-hydroxyphenoxy)phenyl]-α-amino propionic acid, or the p-hydroxy phenyl ether of tyrosine—



desiodo-thyroxin

The 4 iodine atoms in this molecule were presumed to occupy the 3, 5, 3′, 5′ positions, and the correctness of this assumption was definitely proved within the following year by Harington and Barger,[380] who synthesized the tetraiodo-compound of this structure and found it to be identical with thyroxin. In the same article, a brief report by Lyon proved that the synthesized thyroxin possessed the same physiological effect when administered to myxedematous patients as natural thyroxin. The evidence thus appears to be complete that thyroxin possesses the following structure—



Kendall [381] has announced his acceptance of this formula.

The importance of this work of Harington cannot readily be overemphasized. Of particular significance to this discussion is the fact that it relates thyroxin and thyroid activity to tyrosine rather than to tryptophane. As Harington and Barger say: "On general grounds one is almost bound to regard thyroxin as being derived from tyrosine, through the stage of 3-5-diiodotyrosine; assuming this to be the case, it is highly probable that thyroxin is formed in nature by the coupling of two molecules of diiodotyrosine with the loss of one side chain; such a reaction would lead to a compound of the suggested constitution."

[380] Harington, C. R., and Barger, G., *Biochem. J.*, 1927, xxi, 169.
[381] Kendall, E. C., *J. Biol. Chem.*, 1927, lxxii, 213.

3,5-diiodotyrosine has been found as a constituent of albuminoid structures in marine invertebrates. In 1896, Drechsel [382] isolated it from the horny axial skeleton of a Gorgonian coral (*Gorgonia Cavolini*) and named it iodogorgoic acid (Jodgorgosäure). Henze [383] studied the constitution of this substance, but its final identification as 3,5-diiodotyrosine was accomplished by Wheeler and Jamieson [384] in 1905. The iodine complex of sponges was identified as 3-5-diiodotyrosine by Wheeler and Mendel,[385] while Mörner [386] has observed its wide distribution in the skeletons of Anthozoans. It is significant that iodine readily combines with tyrosine under conditions which might be approximated in the animal organism,[387] while it does not with phenylalanine, and that 3,5-diiodotyrosine has been isolated from a variety of artificially iodized proteins by Oswald.[388] It appears that the absorption of iodine by proteins is mainly accounted for by the formation of iodized tyrosine.

Although thyroxin is the only active constituent of the thyroid gland that has been isolated and chemically identified, its physiological action cannot at present be said to account for the manifold phases of thyroid function, as manifested in pathological conditions of the thyroid and the results of thyroidectomy and thyroid feeding. The most characteristic physiological property of thyroxin is its calorigenic activity, and this activity is the one that has received the most investigation.

The most marked effect of thyroxin is obtained from its intravenous administration to patients suffering from complete untreated myxedema. In such patients, 1 mgm. of thyroxin increases the basal metabolic rate an average of 2.8 per cent. According to Boothby and Sandiford: [389]

"By means of small intravenous doses of thyroxin repeated at short intervals it is known that less than 1 mgm. daily is more than sufficient to replace any loss or destruction of thyroxin in the body, although the exact amount has not yet been definitely determined. However, by extrapolation of the descending part of the calorigenic curve of thyroxin it can be estimated that the rate of loss or destruction of the thyroxin usually ranges between 0.2 and 0.4 mgm. daily in a myxedematous subject brought up to a normal basal metabolic rate; total metabolism rather than basal metabolism, however, as well as the concentration of thyroxin

[382] Drechsel, E., *Z. Biol.*, 1896, xxxiii, 90.
[383] Henze, M., *Z. physiol. Chem.*, 1903, xxxviii, 60; 1907, li, 64.
[384] Wheeler, H. C., and Jamieson, G. S., *Amer. Chem. J.*, 1905, xxxiii, 365; 1907, xxxviii, 356. Wheeler and Johns, C. O., *ibid.*, 1910, xliii, 11.
[385] Wheeler, H. C., and Mendel, L. B., *J. Biol. Chem.*, 1909, vii, 1. *Also* Oswald, A., *Z. physiol. Chem.*, 1911, lxxv, 353.
[386] Mörner, C. T., *Z. physiol. Chem.*, 1907, li, 33; 1908, lv, 77, 223.
[387] Oswald, A., *Arch. exptl. Path. Pharm.*, 1908, lx, 126.
[388] Oswald, A., *Beitr. chem. Physiol. Path.*, 1903, iii, 391; *Z. physiol. Chem.*, 1910-11, lxx, 310; 1911, lxxi, 200; 1911, lxxii, 374; 1911, lxxiv, 290; 1915, xcv, 351. *See also* Blum, F. and Vaubel, W., *J. prakt. Chem.*, 1898, lvii, 365.
[389] Boothby, W. M., and Sandiford, I., *Physiol. Revs.*, 1924, iv, 69.

in the tissue probably govern the rate of thyroxin loss. From the thyroxin curves it can further be calculated that 12 to 14 mgms. of active thyroxin must be present in the body in order to maintain the basal metabolic rate at the normal level."

Other calculations by Boothby and Sandiford [390] have shown that in the myxedematous individual, in whom the thyroid gland is totally functionless, the injection of 1 mgm. of thyroxin intravenously produces a total of 1008 cals. The descending limbs of the calorigenic curves of thyroxin show a remarkable tendency to approximate a straight line. In a large proportion of experiments the observations lie within $\pm$ 5 per cent of such a line, and in a few are directly upon it. "This is consistent with an interpretation that the system suffers an essential change of concentration of only one molecular species and that, therefore—the rate of excess heat production is dependent directly upon the concentration of thyroxin . . . in the tissues. The duration of time for the decrease in heat production from the maximum to one-tenth of this amount is . . . for thyroxin between 30 and 70 days."

If thyroxin is given by mouth the height of the metabolic reaction is neither so great nor so constant as when administered intravenously, probably due to its partial destruction in the alimentary canal or its incomplete absorption. Therefore, the amount required daily to maintain the basal metabolic rate of a myxedematous patient is close to 1.6 mgms. by oral dosage, as compared with less than 1 mgm. by intravenous injection.

Boothby and Sandiford have found that thyroxin injected intravenously causes an unmistakable rise in the heat production of normal dogs, but the reaction is neither so great nor so prolonged as in the thyroidless human subject.

Aside from its marked calorigenic effect on animals, particularly on thyroid-less animals, thyroxin has been shown to accelerate the metamorphosis of frog larvæ [391] in a manner similar to thyroid material itself, as first observed by Gudernatsch in 1912. However, Hunt [392] reported that thyroxin did not respond as actively as thyroid material to his acetonitrile test for thyroid. This test is based upon the fact that the resistance of white mice to acetonitrile ($CH_3.CN$) is greatly increased by thyroid feeding. The lesser activity of thyroxin was noted whether it was given orally or intravenously. Hunt concludes, therefore, that thyroxin does not fully represent the physiological activity of

[390] Boothby, W. M., and Sandiford, I., *Proc. Amer. Soc. Biol. Chem.*, 1923, vi, 40.
[391] Romeis, B., *Klin Wochn.*, 1922, i, 1262 (*Chem. Abst.*, 1923, xvii, 2154). Swingle, W. W., Helff, O. M., and Zwemer, R. L., *Amer. J. Physiol.*, 1924, lxx, 208.
[392] Hunt, R., *J. Pharm.*, 1923, xxi, Proc. 199.

the thyroid gland when administered as a drug. However, Miura [393] found that in the proper dosage thyroxin may be even more effective than dried thyroid itself.

Because of the probable close connection between 3-5-diiodotyrosine and thyroxin, it is interesting to note their similarities and dissimilarities in physiological action. The iodized tyrosine has been found to be without calorigenic action, either upon normal or thyroidless animals.[394] In fact, West has reported that diiodotyrosyl-diiodotyrosine and the cyclic anhydride (diketopiperazine) of diiodotyrosine are also without calorigenic effect. However, diiodotyrosine does exhibit an accelerating effect on the metamorphosis of tadpoles,[395] although to a much less extent than thyroxin itself. Romeis [391] found thyroxin to be at least 100 times more effective than diiodotyrosine. Miura [393] reports that diiodotyrosine gives no protection to mice against acetonitrile poisoning in whatever quantity fed.

When diiodotyrosine is fed to animals its iodine is readily split off and largely excreted as inorganic iodides.[396] Oswald [397] has reported that trypsin possesses the capacity of deiodizing this compound.

Tyramine and Phenols

The products resulting from the action upon tyrosine of the bacteria normally occurring in the intestinal tract have been the subject of a large amount of investigation. The fate of these products in the body and their occurrence in blood and urine have also been widely studied. The solution of the general problem of the formation and fate of these tyrosine derivatives has been facilitated, as compared with the same problem with reference to most of the other naturally occurring amino acids, by the fact that they may be readily isolated or detected by more or less characteristic chemical reactions, including color reactions. In general, it may be said that tyrosine is subject to two apparently distinct types of destruction by colon bacilli. It may either be decarboxylated to form tyramine, or it may be deaminized and oxidized to form phenol, possibly according to the following scheme, proposed by Folin and Denis: [398]

[393] Miura, M., *J. Lab. Clin. Med.*, 1921-22, vii, 349.

[394] Strause, S., and Voegtlin, C., *J. Pharm. and Exper. Therap.*, 1909-10, i, 123. Abelin, J., *Biochem. Z.*, 1923, cxxxvii, 161. Beumer, H., and Kornhuber, B., *Münch. med. Wochn.*, 1925, lxxii, 2057; West, R., *Proc. Soc. Exp. Biol. Med.*, 1926, xxiii, 629.

[395] Morse, M., *J. Biol. Chem.*, 1914, xix, 421. Abelin, J., *Biochem. Z.*, 1919, cii, 58; 1921, *Biochem. Z.*, 1923, cxxxvii, 161. Beumer, H., and Kornhuber, B., *Münch. med. Wochn.*, 1925, cxcviii, 151. Abderhalden, E., and Schiffmann, O., *Arch. ges. Physiol.*, 1922, cxcv, 167. Abderhalden, E., *ibid.*, 1924, lxviii, 432.

[396] Abderhalden, E., and Slavu, *Z. physiol. Chem.*, 1909, lxi, 405. Oswald, A., *ibid.*, 1909, lxii, 399; 1910, lxv, 141.

[397] Oswald, A., *Z. physiol. Chem.*, 1909, lxii, 432.

[398] Folin, O., and Denis, W., *J. Biol. Chem.*, 1915, xxii, 309.

$HO.C_6H_4.CH_2.CHNH_2.COOH$, tyrosine
↓
$HO.C_6H_4.CH_2.COOH$, *p*-hydroxyphenylacetic acid
↓
$HO.C_6H_4.CH_3$, *p*-cresol
↓
$HO.C_6H_4.COOH$, *p*-hydroxybenzoic acid
↓
$HO.C_6H_5$, phenol

However, Rhein [399] has found that for one strain of *B. coli* at least, the formation of phenol does not appear to be by way of *p*-hydroxyphenylacetic acid or *p*-cresol, since these compounds were not converted into phenol by this particular organism. The only compounds yielding phenol were tyrosine itself and *o*-hydroxybenzoic acid. Rhein concluded from these results that tyrosine is broken down by oxidation of the β-carbon atom of the alanine side-chain.

The conditions under which different groups of colon bacilli produce tyramine and phenols from tyrosine have been studied by Hanke and Koessler,[400] who found that the ability to destroy tyrosine was possessed by some but not by all strains of bacteria of this general group. They observed that, "in a broadly buffered medium, or one in which the development of acidity is impossible, tyrosine is catabolized to phenol; but only by those bacilli that have the faculty of decarboxylating tyrosine to tyramine in an acid medium. Other colon bacilli do not catabolize tyrosine in alkaline solution." The production of tyramine, like the production of histamine, is viewed as a protective mechanism resorted to "when the accumulation of H-ions within the bacterial protoplasm is incompatible with its normal life processes." It bears no relation to the presence of carbohydrates as such in the medium. Quoting these investigators further:

"In a medium having a solid or semi-solid consistency, such as we find in the large intestine of man, it is possible for areas of acidity and of alkalinity to develop in fairly close proximity due to a difference in chemical composition. Under such conditions, phenols and amines might be produced simultaneously and this seems to be the case. That the normal intestinal contents of man contain phenol is a generally recognized fact . . . histamine is also a normal constituent of human feces."

Tyramine appears to be oxidizable to some extent in the body. Ewins and Laidlaw [401] observed its partial conversion (25%) into *p*-hydroxyphenylethylamine when fed to dogs, or when perfused

[399] Rhein, M., *Biochem. Z.*, 1918, lxxxvii, 123.
[400] Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1924, lix, 867.
[401] Ewins, A. J., and Laidlaw, P. P., *J. Physiol.*, 1910, xli, 78.

through cats' or rabbits' livers. When perfused through the hearts of rabbits, dogs, or cats the amine completely disappeared, but no *p*-hydroxyphenylacetic acid was found. Tyramine is oxidized by tyrosinase.[402]

Like histamine, tyramine possesses a distinct physiological effect,[403] though it is not even approximately as potent pharmacologically as histamine. It is an interesting fact that in its effect upon blood pressure, smooth muscle, and the absorptive powers of the intestinal mucous membrane, tyramine resembles adrenalin, though it is much less potent.[404]

The phenols formed in the intestinal tract are to a large extent absorbed, oxidized or detoxicated, and the residues excreted in the urine. In studying the physiological disposal of absorbed phenols, it has been necessary to analyze blood, urine, and tissues for this class of compounds. Their occurrence in the urine under different conditions, in particular, has been the subject of a large amount of investigation since the pioneer work of Baumann in 1876 and later. The earlier work was concerned largely only with the volatile phenols, *p*-cresol and phenol, and the methods used have been shown to be unsatisfactory by Folin and Denis [405] and by Tisdall.[406] The later work has been done with some modification of the colorimetric method introduced by Folin and Denis. This method, however, as Tisdall points out, is not specific for volatile phenols, even in the absence of uric acid, since the phosphotungstic-phosphomolybdic reagent reacts with non-volatile phenols, such as pyrocatechin, with aromatic hydroxy acids, such as *p*-hydroxyphenyl-propionic, *p*-hydroxyphenylacetic acid, and *p*-hydroxybenzoic acid, with tyrosine, tryptophane, and other indole derivatives, and probably with other compounds present in biological material. The figures obtained with the method of Folin and Denis thus possess quite a different significance from the figures obtained with the older methods, and unfortunately are less definite. In this connection the methods more recently proposed by Hanke and Koessler [407] appear to be promising.

Benedict and Theis [408] were the first to apply the phenol method of Folin and Denis for urine to blood. By determining the uric acid in a separate sample of blood, it was possible to make allowance for this

[402] Abderhalden, E., and Sickel, H., *Fermentforschung*, 1923, vii, 85. Stapp, C., *Biochem. Z.*, 1923, cxli, 42. Wyss, F., Tyrosinase, *Univ. Genève Thèse* 693, 1-52, 1922 (*Chem. Abst.*, 1926, xx, 2169).

[403] Hewlett, A. W., *Arch. Intern. Med.*, 1918, xxi, 411. McCarrison, R., *Indian J. Med. Sci.*, 1923-24, xi, 1137. Tainter, M. L., *J. Pharm. Exp. Therap.*, 1926, xxx, 163.

[404] Hilz, K., *Arch. exptl. Path. Pharm.*, 1922, xciv, 129. Douglas, B., *Compt. rend. soc. biol.*, 1925, xcii, 265.

[405] Folin, O., and Denis, W., *J. Biol. Chem.*, 1915, xxii, 305.

[406] Tisdall, F. F., *J. Biol. Chem.*, 1920, xliv, 409.

[407] Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1922, 1, 235, 271.

[408] Benedict, S. R., and Theis, R. C., *J. Biol. Chem.*, 1918, xxxvi, 95.

blood constituent which is known to react with the phenol reagent. In a number of pathological cases, Theis and Benedict [409] obtained phenol values in human blood ranging from 1.9 to 8.0 mgms. per 100 cc., and averaging 4.7 mgms. These values are expressed in terms of phenol itself. In this investigation no evidence was obtained of the presence in blood of conjugated phenols; in other words, the color intensity produced was not increased appreciably by previous treatment of the blood with HCl. Some evidence was found to the effect that polyphenols account for about one-third of the total phenol content of blood.

Pelkan,[410] using another modification of the Folin and Denis method for phenols in which, in particular, the possibility of loss of volatile phenols was avoided, obtained values for dog and human blood quite comparable to those of Theis and Benedict. However, due to the special precautions taken in his work, Pelkan was able to demonstrate the presence of conjugated phenols in blood, accounting for from 4 to 11 per cent of the total phenols.

Finally, using still another modification of the phosphotungstic-phosphomolybdic acid colorimetric method, Rakestraw [411] has reported much lower values for the total phenols in blood and much higher per cents of conjugated phenols than those obtained in the preceding studies. His values for total phenols in human blood ranged generally between 2 and 3 mgms. per 100 cc., while the conjugated fraction varied widely from 6 to 25 per cent.

From the fact that, if a known amount of phenol or *p*-cresol is ingested, only a variable fraction can be recovered from urine and feces,[412] it has been inferred that the animal body is capable of oxidizing such compounds to a large extent, with the production of substances no longer giving phenol reactions; possibly the oxidation is carried on to complete disintegration. Although some oxidation of such compounds is possible, they are refractory to such a method of disposal and their toxicity necessitates some other method of disposal. While they are to a large extent rapidly excreted in the urine, they are also to some extent detoxicated by conjugation with sulfuric acid or with glucuronic acid.

Pelkan and Whipple [413] were concerned in their experiments with a study of this conjugating process. They observed that when phenols are injected intravenously into animals they are promptly and uniformly distributed to the different tissues of the body. Following such injec-

[409] Theis, R. C., and Benedict, S. R., *J. Biol. Chem.*, 1918, xxxvi, 99.
[410] Pelkan, K. F., *J. Biol. Chem.*, 1922, l, 491.
[411] Rakestraw, N. W., *J. Biol. Chem.*, 1923, lvi, 109, 121.
[412] Tauber, E., *Z. physiol. Chem.*, 1878-79, ii, 366. Jonescu, D., *Biochem. Z.*, 1906, i, 399. Dubin, H., *J. Biol. Chem.*, 1916, xxvi, 69.
[413] Pelkan, K. F., and Whipple, G. H., *J. Biol. Chem.*, 1922, l, 499, 513.

tion, there was a rapid decrease in the concentration of free phenols in the blood and a rapid increase in conjugated phenols. The rise in conjugated phenols usually continued for about 1 hour, and was then followed by a decline as urinary excretion occurred. The conjugated phenols were also uniformly distributed to the tissues. Following the ingestion of phenols (by stomach tube), a different picture was observed. With a sufficiently large dose some free phenols appeared transiently in the blood. The conjugated phenols showed an immediate rise in the first and second hours, followed by a decline due to urinary excretion. The conjugated phenols were rapidly eliminated, probably within 12 hours.

The difference in the blood changes following the two types of phenol administration suggests that the liver is the seat of conjugation. And in fact it was shown, in confirmation of Dubin's earlier work,[412] that liver exclusion by means of the Eck fistula and partial ligation of the hepatic artery, practically eliminates phenol conjugation. It was also observed that the presence of a slight liver injury due to chloroform or phosphorus poisoning may not modify the phenol conjugation. However, extensive liver injury by means of these poisons always lessened phenol conjugation, and if carried to an extreme will reduce phenol conjugation to zero. It appears, therefore, that the liver is the sole organ concerned with this particular detoxication mechanism.

The voluminous literature on the excretion of urinary phenols has been reviewed in the papers of Folin and Denis,[398] Dubin [412] and Tisdall.[406] The volatile phenols of human urine have been found to consist largely of *p*-cresol and phenol. Pyrocatechin has also been found in human urine, while hydroquinone has been isolated from dog urine. Other non-volatile urinary constituents giving the phenol reaction of Folin and Denis include a number of aromatic hydroxy acids derivable from tyrosine by bacterial action. The most significant results of investigations on the excretion of phenols in the urine may possibly be summarized as follows:

1. The phenol excretion may be largely or entirely suppressed by the ingestion of intestinal antiseptics such as calomel. This fact indicates that the urinary phenols are produced entirely in the intestinal tract by bacterial action.

2. The excretion of phenols in the urine varies directly with the level of protein intake and is influenced markedly by irregularities in bowel motility and in defecation. Constipation and intestinal obstruction cause large increases in phenol excretion, and diets which in general promote the growth of putrefactive bacteria in the intestine also pro-

mote phenol excretion.[414] Evidently the excretion of urinary phenols is a measure of the extent of intestinal putrefaction.

3. Generally only one half or even less of the total phenols in urine is conjugated, though this fraction may be increased considerably by the feeding of *p*-cresol or phenol.[412] However, of the volatile phenols, which are the toxic phenols, only a small fraction is excreted in the free state under normal conditions.[406] The volatile phenols account for only 50 per cent or less of the total phenolic substances (expressed as phenol) determined by the Folin and Denis method.

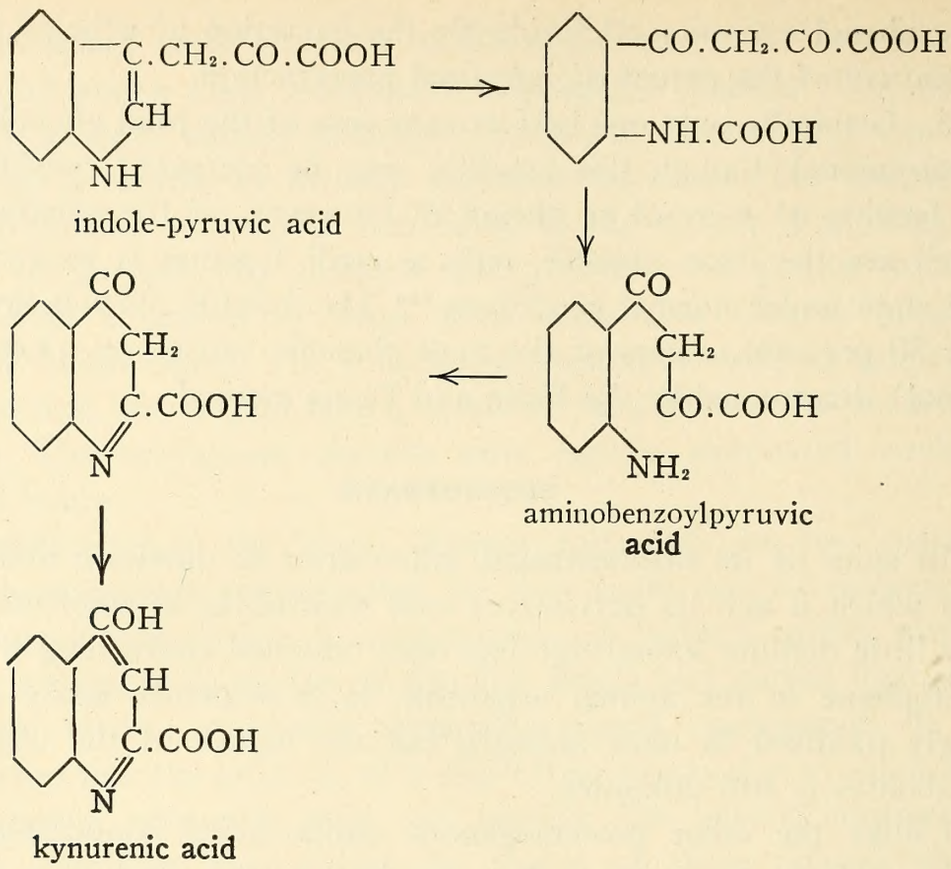
TRYPTOPHANE

In spite of its demonstrated importance in nutrition and the ease with which it and its derivatives lend themselves to colorimetric tests, very little definite knowledge has been obtained concerning the fate of tryptophane in the animal organism. It is evidently easily and completely oxidized in most animals, but the nature of the intermediary metabolites is still unknown.

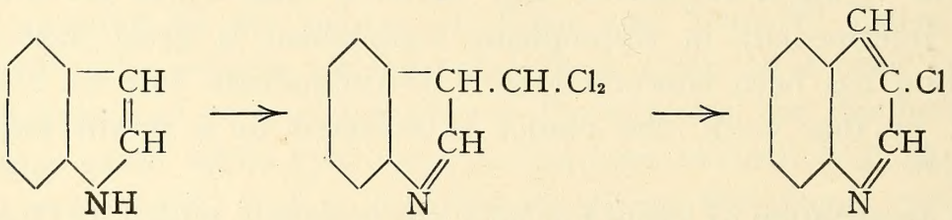
Unlike the other proteinogenous amino acids containing an aromatic nucleus, tryptophane does not lead to the production of homogentisic acid in the alcaptonuric. However, in certain animals (dog, rabbit, rat) a metabolic derivative of tryptophane, kynurenic acid, has been found in the urine and is normally there after tryptophane ingestion. While it cannot be said definitely that kynurenic acid is a normal intermediate in tryptophane metabolism, a great deal of investigation has been concerned with its production. For an historical account of this work, the reader is referred to a recent paper by Robson.[415]

The constitution of kynurenic acid was definitely settled by Homer [416] in 1914 as 4-hydroxyquinoline-2-carboxylic acid. Ellinger and Matsuoka [417] showed that the α-ketonic acid corresponding to tryptophane, indole-pyruvic acid, is also convertible into kynurenic acid in the dog; this may be taken as evidence that tryptophane, like tyrosine, is normally deaminized oxidatively rather than hydrolytically. The conversion of indole-pyruvic acid into kynurenic acid involves the entrance of a carbon atom into the pyrrole ring of the indole nucleus to produce the quinoline nucleus. This conversion is pictured by Ellinger and Matsuoka as follows:

[414] Underhill, F. P., and Simpson, G. E., *J. Biol. Chem.*, 1920, xliv, 69.
[415] Robson, W., *J. Biol. Chem.*, 1924, lxii, 495. *See also* Dakin, H. D., "Oxidations and Reductions in the Animal Body," pp. 95-97.
[416] Homer, A., *J. Biol. Chem.*, 1914, xvii, 509.
[417] Ellinger, A., and Matsuoka, Z. *Z. physiol. Chem.*, 1920, cix, 259.



The remarkable reaction involved in the conversion of the indole into the quinoline nucleus has been observed *in vitro* by Ellinger and others; for example in the production of β-chloroquinoline from indole by the action of chloroform and potassium hydroxide—



Kynurenic acid is formed by the surviving dog's liver, as readily from indole-pyruvic acid as from tryptophane,[418] and its formation in other tissues also is indicated by its occurrence in the urine of Eck fistula dogs.[419] It is extremely resistant to oxidation both in rabbits [420] and in dogs,[421] but in the case of man, when administered by mouth, very little is excreted in the urine.[422] It cannot yet be decided whether kynurenic acid is or is not a normal catabolite of tryptophane.

Dakin [423] could obtain no evidence of the conversion of tryptophane

[418] Matsuoka, Z., and Takemura, S., *J. Biochem.* (*Japan*), 1922, i, 175.
[419] Abderhalden, E., London, E. S., and Pincussohn, L., *Z. physiol. Chem.*, 1909, lxii, 139.
[420] Matsuoka, Z., *J. Biol. Chem.*, 1918, xxxv, 333.
[421] Homer, A., *J. Biol. Chem.*, 1915, xxii, 391.
[422] Ellinger, A., *Z. physiol. Chem.*, 1904-05, xliii, 325.
[423] Dakin, H. D., *J. Biol. Chem.*, 1913, xiv, 321.

into sugar in the phlorhizinized dog nor into acetoacetic acid in the surviving liver. Recently, Matsuoka and Yoshimatsu [424] have reported the production in rabbits, after subcutaneous injection of tryptophane, of considerable amounts of an unknown substance, the constitution of which is being investigated. Attention may also be called to an interesting observation of Eppinger [425] relative to the metabolism of tryptophane. In a patient with melanotic tumors, the excretion of melanogen in the urine was increased threefold when 1.5 grams of tryptophane were added to the diet. Tyrosine and phenylalanine produced no effect. The melanogen separated from the urine appeared to be a pyrrole derivative.

In feeding experiments upon rats, Asayama [426] could obtain no evidence that kynurenic acid effectively supplemented a tryptophane-free diet, while, in similar experiments, Sure [427] found that gelatin supplemented with indole and alanine was no more effective than gelatin alone in covering the protein requirements of rats.* However, experiments of this type, unless conducted in such a way that control and test rations are consumed in equal or equivalent amounts, cannot yield a decisive answer to the problem of the synthetic powers of the animal body.

The supposed relation of tryptophane to thyroid function has been negatived by Harington's demonstration that thyroxin, the only known hormone in the gland, is not a tryptophane derivative, but is in all probability related to tyrosine. Chang [428] has recently summarized the experimental work concerned with this relation, and shows that it has in fact been inconclusive.

The Putrefactive Products from Tryptophane

By the action of intestinal bacteria upon tryptophane, indole-ethylamine and a series of indole derivatives produced after reductive deamination are formed. Among the latter would be found indole-propionic acid, indole-acetic acid, indole-carboxylic acid, and finally indole itself. β-Methyl-indole (skatole) is also occasionally found in the feces.[429]

The deaminized products of tryptophane putrefaction do not seem to be markedly toxic,[430] and are largely resistant to oxidation in the

[424] Matsuoka, Z., and Yoshimatsu, N., *Z. physiol. Chem.*, 1925, cxliii, 206.
[425] Eppinger, H., *Biochem. Z.*, 1910, xxviii, 181.
[426] Asayama, C., *Biochem. J.*, 1916, x, 466.
[427] Sure, B., *Amer. J. Physiol.*, 1925, lxii, 260.
* "In similar experiments, Jackson (*J. Biol. Chem.*, 1927, lxxiii, 523) was unable successfully to substitute 3-indole aldehyde, or *l*-β-3-indole lactic acid for tryptophane in the rations of growing rats."
[428] Chang, H. C., *Endocrinology*, 1926, x, 188.
[429] Herter, C. A., *J. Biol. Chem.*, 1908, iv, 253.
[430] Underhill, F. P., and Kapsinow, R., *J. Biol. Chem.*, 1922, liv, 717.

body. They appear in the urine in unchanged or only slightly changed form, either in the free condition or combined with sulfuric acid or other substances. Indole and skatole are hydroxylated to indoxyl and skatoxyl, respectively, and appear in the urine mainly as salts of the conjugated sulfuric acid. Indoxyl sulfuric acid is known as indican and the rate of its excretion in the urine is frequently taken as an index to the extent of intestinal putrefaction. Indole-propionic acid is also hydroxylated,[431] while indole-acetic acid and indole-carboxylic acid appear to undergo no change, except for conjugation.

Ewins and Laidlaw [432] have demonstrated that indole-ethylamine, when injected into the animal organism, is excreted as indole-acetic acid conjugated with glycine. This oxidation to the acetic acid derivative also occurs on perfusion of the amine through the surviving liver. Ward has shown that indole-ethyl alcohol is likewise oxidized to indole-acetic acid.[431] The possible association of an endogenous production of indole-acetic acid with certain nervous disorders (dementia praecox) is suggested by the experiments of Ross.[433]

It is of importance to record the observations of Logie [434] on the synthesis of tryptophane by bacteria. The strains which gave the best results were one of *B. coli* and one of *B. friedländer,* both of which appeared capable of growing indefinitely in tryptophane-free media.

[431] Ward, F. W., *Biochem. J.,* 1923, xvii, 905.
[432] Ewins, A. J., and Laidlaw, P. P., *Biochem. J.,* 1913, vii, 18.
[433] Ross, E. L., *Arch. Intern. Med.,* 1913, xii, 112, 231.
[434] Logie, W. J., *J. Pathol. and Bact.,* 1920, xxiii, 224.

CHAPTER VIII

THE SPECIFIC DYNAMIC EFFECT OF AMINO ACIDS.

THE EFFECT OF FOOD ON METABOLISM

It has been known that the ingestion of food increases metabolism since the time of Lavoisier, who noted that the oxygen consumption of a man increases materially after a meal, the increase amounting to as much as 37 per cent. It has also been clearly shown that different foods have very different effects on metabolism. Thus, Magnus-Levy has described experiments in which various foods were given to a dog and the respiratory metabolism determined in short periods each hour after the intake of food. After giving 1800 grams of meat the oxygen consumption rose from 185 cc. to 355 cc. per minute, an increase of almost 100 per cent. The metabolism was maintained at approximately this height from the third to the eighth hour. After giving rice, or rice and cane sugar, the oxygen consumption was increased only 20 per cent, whereas with very large quantities of fat the increase was not more than 10 per cent in 18 hours.

A more precise statement of the effect of the different foodstuffs on metabolism may be obtained from the work of Rubner, who was able to show that, for an intake of 100 calories in each case, the effect of protein on metabolism is measured by an increase of 30.9 calories, the effect of fat by an increase of 12.7 calories, and the effect of cane sugar by an increase of 5.8 calories. Stated in another way, in order to maintain a dog in energy equilibrium at or above the critical temperature, the following amounts of the different nutrients must be given for each 100 cals. of basal (or fasting) metabolism:

Protein, as meat.....	140 cals.
Fat	114 "
Cane sugar	106 "

More recent work reverses the relative "specific dynamic effect" (to use Rubner's term for it) of fats and carbohydrates, ascribing to the latter a greater effect than to the former, though it may be said in general that no definite factors can be assigned to any food nutrient for all conditions.

The predominant specific dynamic effect of protein is noteworthy.

After the ingestion of excessive amounts of meat, the total metabolism of the dog may be increased 100 per cent, an increase comparable to that induced by considerable muscular work. This extra heat production in turn produces a slight increase in body temperature, and a marked increase in respiration and heart action, so that the analogy to the effect of muscular work is pronounced.

The general facts of the case as stated above are beyond dispute and have been repeatedly confirmed. The explanation of the facts, however, has offered many difficulties, and consequently many divergent theories have been put forward. Before reviewing these theories, it may be well to consider the possible factors involved in the increment in metabolism consequent upon the consumption of food.

The Possible Factors Operating

The possible factors entering into the increment in metabolism following the ingestion of food may be summarized as follows:

1. *Mechanical Work.* When food is taken a certain amount of muscular work is involved in reducing it to a proper degree of fineness, in mixing it with the digestive juices, and in transporting it from one end of the digestive tract to the other. The processes of mastication, deglutition, rumination, peristalsis, rhythmic segmentation, etc., must involve the consumption of energy and ultimately the liberation of heat. Evidently the amount of energy expended in these muscular movements will depend primarily upon the "roughness" of the food, the extent to which it is reduced by mastication, and its digestibility.

For example, it has been shown (Barcroft and Shore) that the consumption of oxygen by the organs drained by the portal vein is equivalent to 0.008 cc. to 0.013 cc. per gram of viscera per minute in unfed cats, and to 0.011 cc. to 0.018 cc. in cats fed 18 hours previously. Obviously the difference would be greater if the latter values were taken at the height of digestion, instead of in the last stages of digestive activity.

Besides this digestive work, the ingestion of food by farm animals brings about an increased restlessness, as shown in minor muscular movements. It should be remembered that this factor is included in the increments in metabolism following feeding as determined from experiments on farm animals, whose muscular activities cannot be controlled or measured, except in a purely qualitative fashion. It is a factor which may vary considerably during the course of respiration and calorimetric experiments, a fact which often renders the results obtained of ambiguous significance. On the other hand, with the smaller experimental

animals and with man, incidental muscular movements can be practically eliminated, thus enhancing greatly the significance of results obtained. The increments in metabolism consequent upon food ingestion with such subjects do not include an increase in incidental muscular movements.

2. *Glandular Work.* The digestion of food calls into action the numerous glands lining the digestive tract and pouring their secretions into it. Secreting glands require energy for the production of the chemical constituents of their secretions, whenever these substances are not already present in the blood, or for the production of a greater concentration of blood constituents in their secretions than in the blood itself.

The resting submaxillary gland of the cat consumes about 0.027 cc. of oxygen per gram of tissue per minute. When excited to secretion, the consumption may rise to as much as 0.089 cc. in the same units. From such experimental data it may be calculated that 0.18 cc. of oxygen is necessary for the formation of 0.30 cc. of saliva (Barcroft and Piper). However, it is interesting to note that Bayliss and Hill, even by the use of very delicate calorimetric methods, were unable to detect a formation of heat in the salivary gland. Chauveau and Kaufman have found a diminution of glucose in the blood after passage through the salivary gland of the horse.

Similar results have been found by Barcroft and Shore for the liver. The "coefficient of oxidation" for livers of cats which have been unfed for 36 hours was determined to be from 0.005 cc. to 0.018 cc. per gram of liver per minute; for animals fed 18 hours before the experiment, the values ranged from 0.024 cc. to 0.050 cc.

3. *Chemical Work.* The chemical disintegrations occurring during digestion as the result of enzyme action are all hydrolyses, reversible according to conditions, and therefore involve the liberation of only small amounts of energy. For example, the glucose obtained by the complete hydrolysis of starch contains about 99.2 per cent of the energy of the starch; that is, during the digestion of starch, only 0.8 per cent of its energy is liberated as heat. Approximately the same holds true for proteins and fats.

4. *Bacterial Decompositions.* The extensive bacterial fermentations of carbohydrates occurring in the first three stomachs of ruminants give rise to a considerable amount of heat. The gases formed by these fermentations are CO_2, CH_4, and usually only traces of H_2. It has been computed (Armsby) that 4.5 grams of CH_4 are formed per 100 grams of digested carbohydrates (crude fiber and nitrogen-free extract) in

the case of ruminants. The formation of each gram of CH_4 is said to be accompanied by the evolution of approximately 6.07 calories (Markoff). Thus, for each 100 grams of digested carbohydrates, approximately 27.3 calories of heat are liberated. Assuming the former to be in the form of glucose with an energy value of 3.75 calories per gram, it may be estimated that in the digestion of carbohydrates by ruminants for each 100 calories absorbed, 7.21 calories are dissipated as heat. In the case of the horse, the methane fermentation occurs largely in the cecum and colon and is probably not so extensive as the above figures indicate. With pigs, fermentations are generally even less extensive, while for man and the carnivora, this source of heat is probably negligible.

The bacterial putrefaction of proteins and protein derivatives occurring in the large intestines of all animals are undoubtedly accompanied by the evolution of heat, though no attempt has been made to estimate the amount of heat liberated. Probably it is not considerable.

5. *Intermediary Metabolism.* As the products of digestion are absorbed and carried by the blood to the tissues, they undergo more or less profound changes preparatory to final utilization. The amino acids are deaminized, leading to the synthesis of urea and non-nitrogenous acids destined to be stored as glycogen or fat, or to be oxidized directly. The fatty acids are desaturated and built up into the phosphatide molecule. The monosaccharoses are synthesized into glycogen or fat, or transformed into glucose, which in turn is split into three-carbon molecules, from which point its oxidation begins. These various transformations in the assimilation of the food undoubtedly involve energy transformations, and may conceivably account for a certain fraction of the increment in heat production consequent upon the taking of food.

6. *Direct Stimulation of Metabolism.* It has been recently shown, especially by the researches of Gigon, Lusk, and Grafe, that a very important factor in the increase in heat production following the taking of food is a direct stimulation of the tissue cells brought about by the increase in the concentration of nutrient material in the blood and intracellular lymph following the absorption of the end-products of digestion from the intestinal tract. This factor will be fully discussed below.

The "Work of Digestion"

Von Mering and Zuntz believed that the increase of metabolism after the ingestion of food was due entirely to the increased activity of the intestinal tract, a contention which has been consistently maintained

in the many papers from Zuntz' laboratory. The evidence supporting this view, consisted, first, of the fact that the increase began very promptly (within 30 minutes) after a meal, which was interpreted to mean that it could hardly be due to metabolic activity or to any effect of the absorbed food upon metabolism, but that it must arise, in large part at least, from the activity, motor and secretory, of the digestive organs. In the second place, support for the theory was found in the results of specific investigations upon the point reported by Zuntz and von Mering. They found that glycerine, sugar, egg-albumin, purified peptones, and the sodium salts of lactic and butyric acids, when injected into the circulation caused no material increase in the consumption of oxygen, while some of them do increase metabolism when given by mouth. They, therefore, conclude that the effect of the ingestion of food upon metabolism is due chiefly to the expenditure of energy required in its digestion. The accuracy of these injection experiments is open to question in view of recent work, and, in any case, the pertinence of much of this evidence to the question at issue is not clear in the light of what we now know of the extent of the digestive processes. The theory signally fails to account for the fact that protein stands foremost among the food nutrients in its effect on metabolism, exerting an effect 5 or 6 times that of carbohydrates.

The results of recent work have thoroughly discredited the importance of digestive work in accounting for the increase in metabolism after the ingestion of food. Thus, Benedict and Emmes [1] demonstrated that saline cathartics and agar-agar when administered to men had no measurable effect on metabolism, in spite of the increased intestinal motility. Lusk has shown [2] in confirmation of earlier work, that Liebig's extract of meat, which is known to stimulate a flow of digestive juice in both stomach and pancreas, had no effect upon heat production. However, in ruminants the heat produced from the bacterial fermentations occurring in the digestive tract is considerable.

THE EFFECT OF EXOTHERMIC REACTIONS IN INTERMEDIARY METABOLISM

The question of the effect of the ingestion of food on metabolism has been investigated in great detail by Rubner, who concluded that digestive work had little to do with it, but that the preliminary cleavages and oxidations to which the absorbed nutrients were subjected in metabolism before final utilization might entirely account for this effect. Thus, the profound effect of protein food was due, in his opinion, to the free heat liberated in the conversion of the absorbed products of

[1] Benedict, F. G., and Emmes, L. E., *Amer. J. Physiol.*, 1912, xxx, 197.
[2] Lusk, G., *J. Biol. Chem.*, 1912, xiii, 155.

digestion into glucose and the cleavages involved in the elimination from the body of the nitrogenous waste products.

In confirmation of this view, Rubner [3] attempted to show that an increased catabolism of body protein in inanition or in phlorhizin diabetes had about the same quantitative effect on the heat production as an increased catabolism of dietary protein. The calculations, however, are not particularly convincing, especially with reference to inanition, and need not be repeated here. Rubner's theory resulted more as a revulsion against the older theory of Zuntz that the heating effect of food was due solely to the work of digestion, absorption and excretion, than as a probable interpretation of certain specific experimental data.

THE FOOD NUTRIENTS AS DIRECT STIMULI TO CELLULAR METABOLISM

During the last fifteen years, Lusk and his associates have carried out elaborate investigations on the specific dynamic effects of the different nutrients. These investigations have led to a more precise understanding of the relative importance of the factors involved, and are worthy of detailed consideration. Although this chapter is primarily concerned with the specific dynamic effects of the amino acids, a review of the situation with respect to the other nutrients is essential to an understanding of the distinctive effect of the end-products of protein digestion.

In this work, which was performed entirely upon dogs, the metabolism was measured indirectly by hourly determinations of the oxygen absorbed, the carbon dioxide eliminated, and the urinary nitrogen excreted, and directly by hourly determinations of the heat emitted. These experiments demonstrate convincingly the inadequacy of the older theories, and bring forward final proof that the predominating factor is a direct stimulus to cellular metabolism brought about by the inflow of nutrients from the gastro-intestinal tract.

THE SPECIFIC DYNAMIC EFFECT OF CARBOHYDRATES

Two of Lusk's experiments [4] illustrate well the effect on the basal metabolism of the ingestion of glucose. The results are given in Table 33.

In the first experiment, the ingestion of 50 grams of glucose dissolved in 150 cc. of water caused a rise in the metabolism from the basal level of 16.2 calories to 19.6 in the second hour, which level was very nearly maintained during the third and fourth hours, and then fell to the basal value in the fifth hour. It is apparent from the non-protein

[3] Rubner, M., "Die Gesetze des Energieverbrauchs bei der Ernährung," 1902, p. 373.
[4] Lusk, G., *J. Biol. Chem.*, 1912, xiii, 27; *ibid.*, 1915, xxii, 15.

TABLE 33. *The Influence of Glucose on Metabolism.*

Dog No.	Exper. No.	Glucose Ingested	Hourly Basal Metabolism	Metabolism in Calories per Hour: Hours After Food 2nd	3rd	4th	5th	6th
II	20+12	50 g.	16.2 cals.	19.6	19.5	18.2	16.6	...
Increase over basal metabolism in per cent				21	20	21	1	...
Average non-protein R. Q.				0.98	0.96	1.03	0.83	...
XIV	5	70 g.	21.5 cals.	29.0	28.0	24.7	21.2	20.0
Increase over basal metabolism in per cent				35	31	23	...	...
Average non-protein R. Q.				1.03	1.04	1.23	0.92	1.06

respiratory quotients that carbohydrate combustion dominated through the fourth hour. In the fifth hour the quotient fell. The maximum increase in the intensity of metabolism amounted to 21 per cent. The total increase during the four hours was 9.1 calories. Since 50 grams of glucose contain 184.6 calories, the ingestion of 100 calories of glucose would be followed by a specific dynamic effect of 4.9 calories.

The second experiment shows substantially the same thing: An increase in metabolism reaching its maximum in the second hour or before, and a rapid decrease to the basal level in the fifth hour, the metabolism being essentially carbohydrate in character. In this case, the total increase in metabolism during the second, third, and fourth hours amounted to 6.6 calories per 100 calories of ingested glucose.

A better insight into the changes in metabolism induced by the ingestion of glucose is obtained from the following analysis of the heat production, first, in a basal metabolism period, and second, in the second and third hours after the ingestion of 70 grams of glucose by the same animal.

TABLE 35. *Analysis of the Heat Production During the Second and Third Hours After the Ingestion of 70 Grams of Glucose.*

	Average R. Q.	Protein	Heat Production in Calories from Carbohydrates: Oxidation	Conversion to Fat	Fat	Total
		cals.	cals.	cals.	cals.	cals.
Basal metabolism	0.87	3.07	11.67	0.00	8.05	22.79
Second and third hours after taking 70 g. glucose	1.02	2.78	27.89	0.19	0.00	30.86

Evidently the increased heat production was brought about entirely by an increased combustion of carbohydrates.

Lusk therefore concludes that the specific dynamic effect of the monosaccharoses, and presumably of all higher carbohydrates that yield monosaccharoses on digestion, is "due to the presence of a greater

amount of free diffusible carbohydrate than is present when there is no absorption from the intestines. When this plethora subsides with the cessation of absorption from the intestines, the glycogenic function regulates the composition of the body fluids and the metabolism falls to the basal level."

THE SPECIFIC DYNAMIC EFFECT OF FATS

The effect of the ingestion of 75 grams of fat on the hourly heat production of a dog (No. III) with a basal metabolism of 19.9 calories per hour, is shown in the following figures:

TABLE 36. *The Influence of 75 Grams of Fat (694 Calories) on the Basal Metabolism.*

Hourly Basal Metabolism	Metabolism in Calories per Hour—Hours After Food										Total Excess Calories
	2	3	4	5	6	7	8	9	10	11	
19.9	21.4	22.1	24.8	24.5	25.9	23.7	23.8	21.3	20.4	17.9	28.8
Increment in %	7	11	25	23	30	19	19	7	2	...	...
Hourly R. Q...	0.80	0.80	0.79	0.79	0.78	0.79	0.78	0.78	0.71	0.84	...

This experiment [4] shows that after the ingestion of 75 grams of fat the heat production gradually rises till the sixth hour to a maximum 30 per cent above the basal metabolism, and then falls gradually again to the basal level, which is reached about 10 hours after the fat has been taken. In comparing the specific dynamic effect of fat with that of glucose, it is evident that the latter reaches its highest intensity much sooner than the former, and that it is much less persistent. This is also true of the relative rates of absorption. With carbohydrates absorption occurs much sooner and runs its course in a much shorter time. Therefore, the specific dynamic effects of carbohydrates and of fats follow, roughly at least, their respective rates of absorption.

In the above experiment, the total increase in heat production from the second to the tenth hour inclusive amounted to 28.8 calories, or 4.1 per cent of the 694 calories of energy contained in the ingested fat. The average respiratory quotient of the basal metabolism of the dog was 0.84, and in four experiments after fat ingestion, of which the above figures are the averages, the respiratory quotient averaged 0.79. It appears, therefore, that the administration of fat causes a relative increase in the metabolism of fat itself. This is clearly brought out from the following analysis of the heat production of two dogs, first, during a basal metabolism period, and, second, during the fifth and sixth hours after the ingestion of 75 grams of fat, when the specific dynamic effect of the latter was at its height:

TABLE 37. *Analysis of the Heat Production During the Fifth and Sixth Hours After the Ingestion of 75 Grams of Fat.*

	Average R. Q.	Hourly Heat Production in Calories from: Protein	Carbohydrates	Fat	Total
Dog III:					
Basal metabolism	0.82	3.53	6.60	9.04	19.17
Metabolism in 5th and 6th hours after fat intake.....	0.77	3.39	4.20	18.65	26.24
Dog XIV:					
Basal metabolism	0.83	3.42	8.64	10.31	22.37
Metabolism in 5th and 6th hours after fat intake.....	0.81	3.18	7.57	13.81	24.56

In the experiment on Dog III, the average increase in heat production during the fifth and sixth hours after the ingestion of fat over the hourly basal metabolism was 7.07 calories, while the increase in heat production from the oxidation of fat was 9.61 calories. In the experiment on Dog XIV, these values were, respectively, 2.19 and 3.50 calories. It appears, therefore, that the administration of fat to an animal increases the metabolism at the expense of the ingested fat, and here, as with carbohydrates, the specific dynamic effect appears to be due to a relative excess (a plethora) of fat in the nutrient fluids surrounding the cells. In the hours following feeding, we have thus, superimposed upon the basal metabolism, a metabolism due to plethora, induced by an increased quantity of carbohydrate and fat metabolites in the blood and lymph on account of absorption from the intestine. This metabolism of plethora is analogous to the increase in heat output of a furnace induced by adding more fuel to the fire.

THE SPECIFIC DYNAMIC EFFECT OF PROTEIN

Of all the food nutrients, protein has the most pronounced effect upon metabolism. For example, Lusk [5] gave a dog weighing 13.5 kg. with a basal metabolism of 22.3 calories per hour, 1200 grams of meat. The heat production rose to 36 calories in the second hour and to 42 calories in the third. A level of approximately 40 calories, about 80 per cent above the basal level, was maintained through the tenth hour after feeding. In the fourteenth hour it had fallen to 37 calories and then remained at 30 calories up to the eighteenth hour, falling rapidly to 25 calories in the twenty-first hour.

While the heat production in this experiment attained its maximum in the third hour after feeding, the urinary nitrogen resulting from the

[5] Lusk, G., *J. Biol. Chem.*, 1912, xii, 349.

metabolism of the protein digestion products of the meat did not reach its maximum until the fifth or sixth hour, when a level of 1.9 to 2.0 grams of nitrogen per hour (basal level, 0.32 gram per hour) was reached; this level was maintained through the tenth hour, after which the nitrogen elimination slowly fell, until at the twenty-first hour, the last hour of observation, the excretion amounted to 0.64 gram. It may be calculated that for every 100 calories arising from protein metabolism (as estimated from the urinary nitrogen figures), the total metabolism was increased 45 calories. This marked effect is to be compared with the effects of the metabolism of 100 calories as fat or carbohydrate; in these cases the total metabolism increases only 4 to 7 calories.

The points to be particularly emphasized in this experiment are: (*a*) The prompt increase in heat production following the ingestion of protein, an increase which probably started in the first hour after feeding. It will be remembered that at this time the products of protein digestion, the amino acids, have apparently not undergone any considerable catabolism in the tissues. They have simply been transported from the intestines to the tissues, by which they have been absorbed and temporarily stored. Not until one to one and one-half hours after feeding does the increase in urea of blood and urine indicate that deamination has started. Therefore, this initial and rapid increase in heat production is probably at the expense of other nutrients than the incoming amino acids, and the rise in the respiratory quotient generally noted in the second and third hours after protein ingestion would indicate that the combustion of carbohydrate has been relatively more intense. The second fact to be noted is (*b*) the greatly prolonged effect of the meat meal, lasting for more than 21 hours. It may be concluded that intestinal work is not an important factor in the specific dynamic effect of protein, because a high level of metabolism is maintained in the fourteenth hour, though three-quarters of the nitrogen in the protein administered has been absorbed and excreted in the urine, and the work of the intestinal canal must have been largely if not entirely completed.

THE SPECIFIC DYNAMIC EFFECT OF AMINO ACIDS

The amino acids resulting from the digestion of protein have also marked effects on metabolism. Lusk has found that the effect varies, depending upon the particular amino acid investigated. The two simplest amino acids, glycine and alanine, were found to have the most pronounced effect of the five investigated. Some typical experiments [6] on

[6] Lusk, G., *J. Biol. Chem.*, 1915, xx, 555.

the effects following the ingestion of these amino acids gave the results tabulated below. The subject of the experiments, Dog III, had a basal metabolism of 16.7 calories per hour.

TABLE 38. *The Heat Production of Dogs Following the Ingestion of Glycine and Alanine.*

Hours After Taking	Average Results of Exps. 56 and 57. Twenty Grams of Glycine Ingested: Computed Heat Production	Increase Over Basal		Respiratory Quotient	Average Results of Exps. 41 and 43. Twenty Grams of Alanine Ingested: Computed Heat Production	Increase Over Basal		Respiratory Quotient
	cals.	cals.	per cent		cals.	cals.	per cent	
2	25.0	8.3	50	0.97	22.1	5.4	32	0.94
3	22.6	5.9	35	0.93	23.0	6.3	38	0.90
4	21.5	4.8	29	0.88	20.8	4.1	25	0.88
5	21.4	4.7	28	0.82	20.1	3.4	20	0.86
6	22.0	5.3	32	0.81	18.0	1.3	8	0.90
7	21.1	4.4	26	0.83				

Evidently glycine has a stronger effect upon metabolism than alanine, though some experiments of Lusk gave results showing inconsiderable differences.

Another point of great importance in these experiments, and others of a similar character, is the high respiratory quotients obtained in the second and third hours after the ingestion of amino acids. The average respiratory quotients of the basal metabolism experiments was 0.88. In the case of glycine, an increase in respiratory quotient from 0.88 to 0.97 may indicate an increased combustion of either glycocoll or glucose, since both of these substances have a respiratory quotient of 1. In the case of alanine, however, a similar, though perhaps slightly less, increase occurs, though alanine has a respiratory quotient of 0.83. In the latter case it appears, therefore, that the presence of alanine in the tissues, brought to them from the intestines by the blood, has stimulated the combustion of glucose.

Leucine and tyrosine were found by Lusk to have only an inconsiderable effect upon heat production,[2] while glutamic and aspartic acids [7] exerted no appreciable effect.* The inactivity of the dibasic amino acids in this respect is rightly interpreted to mean, in contradiction of Rubner's theory, that the processes of deamination and urea formation

[7] Atkinson, H. V., and Lusk, G., *J. Biol. Chem.*, 1918, xxxvi, 415.

* More recently Rapport and Beard (*J. Biol. Chem.*, 1927, lxxiii, 299) observed a greater specific dynamic effect for phenylalanine than that usually obtained for glycine, an effect for tyrosine comparable to that for alanine, and no specific dynamic effect for valine whatever. Wilhelmj and Bollman (*J. Biol. Chem.*, 1928, lxxvii, 127) have confirmed the great heating effect of alanine, glycine and phenylalanine following intravenous, instead of oral, administration. Expressed as calories of extra heat per millimol of amino acid, the specific dynamic effect of these three amino acids proved to be in the approximate ratio of 1 to 1.3 to 2.0.

have nothing to do with the specific dynamic effect of protein. In this connection it has been found that the administration of urea itself and its subsequent complete elimination by the kidneys is without influence upon metabolism; hence, increased kidney activity cannot be assigned an appreciable rôle in the specific dynamic action of protein. The same significance is attached to the inactivity of Liebig's extract of beef on metabolism. The demonstrated potency of meat extractives in stimulating a flow of digestive juice in both stomach and pancreas, considered in connection with the failure of these substances to increase heat production, indicates that increased activity of these glands also is not a considerable factor in the increase in heat production following meat ingestion.

The conclusion that the specific dynamic effect of amino acids is due to a stimulus exerted directly upon cellular metabolism is in agreement with the experiments of Herzog and Saladin [8] on the behavior of the mold, *Penicillium glaucum,* towards amino acids. These investigators report that the addition of leucine to a culture of this mold led to an increased production of carbon dioxide, the over-production being much greater than could be accounted for by the complete oxidation of the quantity of leucine that disappeared. Other amino acids gave similar effects.*

THE PROCESSES INVOLVED IN THE SPECIFIC DYNAMIC ACTION OF AMINO ACIDS

The attempt to factor the heat production of an experimental animal following the administration of an amino acid, into heat produced from carbohydrate, fat, and amino acid, in order to throw significant light upon the nature of the processes involved in the specific dynamic effect, seems futile, because no reliable method of measuring variations in amino acid or protein catabolism is available. The excretion of urinary nitrogen cannot be so used, since, following deamination of amino acids, there is a piling up of urea in blood and tissues, so that there is a considerable lag between its formation and its secretion. Csonka [9] has

[8] Herzog, R. O., and Saladin, O., *Z. physiol. Chem.,* 1912, lxxiii, 302.

* Other physiological effects of amino acids that may be related to their calorigenic effect may also be mentioned. Burge (*Amer. J. Physiol.,* 1918, xlvii, 351) has reported that the administration of glycine and alanine *per os* causes an increase in blood catalase, while that of glutamic and aspartic acid does not; also that a high protein dietary increases the catalase content of the entire body (*ibid.,* 1923, lxiii, 545). Burge, Wickwire, Estes and Williams (*J. Biol. Chem.,* 1927, lxxiv, 235), observed that certain optically active amino acids (including *d*-glutamic acid and *l*-aspartic acid) greatly accelerate the sugar metabolism of paramecia, while racemic amino acids and glycerine do not. Ort and Bollman (*J. Amer. Chem. Soc.,* 1927, xlix, 805) demonstrated that cystine, glycine, alanine, phenylalanine, leucine, histidine, and valine catalyze the oxidation of glucose by hydrogen peroxide, while glutamic acid, aspartic acid, and tyrosine do not, while Witzemann (*ibid.,* 1927, xlix, 987) found that glycine and certain glycolates catalyze the oxidation of butyric acid with H_2O_2 to a marked extent.

[9] Csonka, F. A., *J. Biol. Chem.,* 1915, xx, 539.

suggested that the rate at which glycine and alanine are metabolized in the normal dog may be measured by the rate of excretion of extra glucose in the urine following the administration of these amino acids to phlorhizinized dogs, because under these conditions the extra glucose is eliminated more rapidly than the urea resulting from deamination. Lusk has accepted this criterion of amino acid metabolism in his later work,[6] and by its use has attempted to show that the intensity of the extra heat production following the ingestion of these amino acids runs closely parallel with the intensity of their catabolism. However, it is questionable whether this method of measuring amino acid catabolism is reliable, for the following reasons:

1. There is no convincing theoretical justification for assuming that the rate of excretion of extra glucose in the phlorhizinized animal following amino acid administration would parallel the catabolism of these amino acids in the normal animal. The phlorhizinized animal is in an unmistakable pathological condition. To assume that any of its processes are occurring at a normal rate seems unwarranted. Furthermore, the basic assumption of the method implies that conversion of glycogenic amino acids into glucose is an obligate step in their normal metabolism.*

2. It appears from the experiments of Lusk and Csonka that phlorhizin speeds up metabolism to a considerable extent. The basal heat production of a dog is increased from 20 to as much as 70 per cent by phlorhizin administration, while the endogenous protein catabolism is increased two or three fold. A comparison of the heat production following glycine administration in the phlorhizinized as compared with the normal dog shows that in the former case the rise in metabolism is more abrupt and less prolonged, the maximum being attained sooner and the effect disappearing quicker. The comparative rates of excretion of nitrogen in the urine in the two cases leads to the same general conclusion, as the data of Table 39 show.

From these figures it appears that in the phlorhizinized dog after the ingestion of 20 grams of glycine containing 3.73 grams of nitrogen, 67.8 per cent of the nitrogen were excreted in the six hours following. In the normal dog after taking 25 grams of glycine containing 4.66 grams of nitrogen, only 27.4 per cent of the nitrogen was excreted in the first six hours. Also in the latter case, the maximum excretion of

* Nash and Benedict (*J. Biol. Chem.*, 1923, lv, 757) have obtained direct evidence against this assumption. They found that when a mixture of glucose and urea is ingested by a phlorhizinized dog, the sugar is excreted in the urine more rapidly than the urea. The phenomenon is similar to that observed when meat or proteins are fed to phlorhizinized dogs, and discredits the interpretation of a more rapid production of sugar than of urea in amino acid catabolism.

TABLE 39. *A Comparison of the Rates of Excretion of Extra Ntirogen in the Urine Following the Ingestion of Glycine, in Phlorhizinized and in Normal Dogs.*

Hours After Taking	Csonka's Experiment on Dog V. Phlorhizinized. 20 Grams of Glycine: Extra Nitrogen in Urine		Lusk's Experiment on Dog II. Normal. 25 Grams of Glycine: Extra Nitrogen in Urine	
	grams	per cent	grams	per cent
1.....	0.28	11.1	0.088	6.9
2.....	0.44	17.4	0.192	15.1
3.....	0.55	21.7	0.139	10.9
4.....	0.59	23.3	0.231	18.1
5.....	0.39	15.4	0.323	25.3
6.....	0.28	11.1	0.302	23.7
Total.....	2.53	100.0	1.275	100.0
	67.8 per cent		27.4 per cent	

extra nitrogen in the urine was much later deferred than in the former case. Obviously the metabolism of glycine is very greatly speeded up by phlorhizin.

3. Lusk's calculations of the total heat production of his dogs would be little affected, regardless of the particular method of measuring the rate of amino acid metabolism, since the physiological fuel value of amino acids does not vary greatly from that of glucose, but the number of computed calories derived from the oxidation of the individual food nutrients would be greatly affected, as would also the calculation of the non-protein respiratory quotient. In his latest publications in which he used the method above explained for measuring amino acid metabolism, extremely improbable non-protein respiratory quotients were obtained in his alanine experiments in the first few hours. For the second and third hours after alanine ingestion, the calculated non-protein respiratory quotients are all greater than one, and for the second hour, they are extraordinarily high, *i.e.*, 1.27, 1.50, 1.11 and 1.14. Coefficients as high as these were rarely obtained, even after the ingestion of large amounts of glucose, and indicate a considerable formation of fat from carbohydrate. It is, however, improbable that a synthesis of fat would occur at a time when the body is under the influence of a powerful stimulation, and in consequence the heat production is increasing rapidly. Evidently they are in error, and the error lies in assuming for the early hours a greater metabolism of alanine, with a respiratory quotient of 0.83, than actually occurred. In fact, the total respiratory quotients for these hours indicate an increased combustion of carbohydrate, although the computed non-protein calories in Lusk's tables indicate an actual decrease in the oxidation of both carbohydrates and fats. A similar error

is not evident in the glycine experiments, since the respiratory quotients of glycine and glucose are identical.

THE SPECIFIC DYNAMIC EFFECT OF AMINO ACIDS IS QUALITATIVELY DIFFERENT FROM THAT OF GLUCOSE AND FAT

Although the oxidation processes following the ingestion of amino acids cannot be analyzed as satisfactorily as the processes involved in the specific dynamic effect of fats and sugars, definite evidence has been secured by Lusk that the amino acid stimulus may be qualitatively as well as quantitatively different from the metabolism of plethora due to the inflow of sugar molecules from the intestinal tract. Thus, the ingestion of 70 grams of glucose by a phlorhizinized dog was found [6] not to increase the heat production, although in the normal dog an increase of 35 per cent over the basal was obtained in the second, third, and fourth hours after such administration. Evidently a metabolism of plethora does not supervene if the organism is unable to utilize the sugar by reason of phlorhizin poisoning. However, an ingestion of 12.5 to 20 grams of glycine and alanine produced a distinct rise in the metabolism of the phlorhizinized dog, although no oxidation of the amino acids could have occurred, since they were quantitatively excreted in the urine as glucose and urea. Apparently the amino-acid stimulus is not due only to a plethora of oxidizable molecules in the tissues. The conclusiveness of these phlorhizin experiments, however, is somewhat impaired by the very poor agreement obtained between the results of direct and indirect calorimetry; in fact, the conclusions are based largely upon the indirect estimates, and, in two cases of amino acid feeding (Exps. 28 and 70), are not supported by the direct calorimetric estimates. It may also be mentioned that Benedict and Joslin [10] have reported a marked increase (30 per cent) in the basal metabolism of a patient suffering from severe diabetes after the administration of 100 grams of fructose, although the respiratory quotient was not raised. It is unfortunate that most of Lusk's experiments on the effect of glucose and fructose on the metabolism of phlorhizinized dogs were concerned with quantities of these sugars (10 gram portions) that are without appreciable effect on the metabolism of normal dogs.

A more clear-cut demonstration of a qualitative difference between the specific dynamic effect of glucose and of amino acids was obtained by Anderson and Lusk [11] in experiments on dogs at rest and at work on a treadmill. The results reported show clearly that when mechanical

[10] Benedict, F. G., and Joslin, E. P., "Metabolism in Severe Diabetes," Carnegie Institution of Washington, Publ. No. 136, 1912, p. 69.
[11] Anderson, R. J., and Lusk, G., *J. Biol. Chem.*, 1917, xxxii, 421.

work is accomplished during the hours following a large ingestion of glucose (70 grams), the metabolism rises to about the same height as when the same amount of work is done during a period when the gastro-intestinal tract is free from food. In fact, curiously enough, the energy expenditure for the movement of one kilogram of body weight one meter of horizontal distance was about 5 per cent less after glucose ingestion than in the post-absorptive period. On the other hand, after the ingestion of meat (700 to 750 grams) or of alanine (20 grams), there is a most exact summation of the increment in heat production due to specific dynamic action and that due to muscular activity. Evidently when glucose is used in metabolism as rapidly as it is transported from the intestinal tract, no plethora of oxidizable material accumulates in the tissue fluids and consequently no stimulus to cellular activity results. With amino acids, however, the characteristic stimulus to metabolism appears to be quite independent of variations in energy requirements and, hence also, of a plethora of oxidizable material. It may be questioned, however, in view of the work of Hill and Meyerhof and their associates on the source of muscular energy, whether this difference may not be merely an expression of the fact that glucose is directly used for muscular work, while amino acids are not.

CHEMICAL COMPOUNDS RESPONSIBLE FOR THE STIMULATING EFFECT OF AMINO ACID INGESTION

In deciding what chemical substances are responsible for the powerful stimulating effect of proteins and amino acids, two possibilities have been considered by Lusk: first, the intact amino acids themselves, and, second, the non-nitrogenous products of their deamination, *i.e.*, keto-acids or hydroxy acids. Lusk's own work has indicated that the nitrogenous products of deamination, ammonium salts and ultimately urea, do not appreciably affect cellular metabolism as measured by heat production.

In his earlier publications, up to April of 1915, Lusk was inclined to ascribe to the unchanged amino acids the cell stimulation following protein or amino acid ingestion. Later, however, he changed his views in favor of the keto or hydroxy acids formed on deamination, on the following arguments: [12]

1. One of Rubner's experiments on a dog [3, p. 256] indicated that when ingested protein is retained in the body it exerts no specific dynamic effect. If this is so, the absorption of amino acids by the tissues can hardly be the cause of their specific dynamic effect. However, there

[12] Lusk, G., "The Science of Nutrition," 1917, p. 245.

is no assurance that the muscular activity of the dog was sufficiently well controlled to permit an exact interpretation of the experimental results obtained: there is, in fact, a definite suspicion that uncontrolled factors were operating, since an ingestion of 720 to 760 grams of meat scarcely affected heat production, although less than half of its nitrogen was retained in the body. An experiment of Hoobler's on the feeding of a high-protein diet to an infant [13] is also cited as supporting the same conclusion, but the calorimetric data chosen for comparison were obtained in short observational periods at a definite time after feeding, while the protein metabolism data refer to an entire day. Whether the latter can justifiably be used in interpreting the former may be questioned, particularly in view of the unaccountable irregularity among the calorimetric data of other periods of the experiment.

2. According to experiments by Van Slyke and Meyer [14] and by Wishart,[15] there is no accumulation of amino acids in the tissues after the ingestion of meat in large quantities; hence, the specific dynamic effect of the meat cannot be due to the intact amino acids. However, a critical analysis [16] of the data in these two investigations detracts considerably from the force of the conclusions drawn, mainly because the data are statistically insufficient to warrant definite interpretation in view of the wide variability encountered among different experimental animals. Furthermore, other investigators have noted distinct increases in the non-protein nitrogen of tissues following protein feeding,[17] which are not accounted for by increases in ammonia or urea. It must be considered also, as Seth and Luck point out,[18] that numerous observations have established the fact "that following the ingestion of a protein or an amino acid diet, the amino-N content of blood remains at a high level for a considerable period, in spite of the rapid absorption by the tissues. This fact indicates clearly that, under the conditions described, a fresh and continuous supply of amino-N reaches the tissues during a period of several hours. Therefore, even though there may not be a static accumulation of amino acids in the tissues, there is no reason to assume that the abundant supply of constantly circulating amino acids is not capable of stimulating the tissue cells to a higher level of metabolism."

3. Lusk's third argument is based upon a point already discussed, *i.e.*, that the specific dynamic effect of the amino acids parallels their

[13] Hoobler, B. R., *Amer. J. Dis. of Children*, 1915, x, 153.
[14] Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 1913-14, xvi, 231.
[15] Wishart, M. B., *J. Biol. Chem.*, 1915, xx, 535.
[16] Mitchell, H. H., *J. Biol. Chem.*, 1918, xxxvi, 501.
[17] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xii, 141.
[18] Seth., T. N., and Luck, J. M., *Biochem. J.*, 1925, xix, 366.

metabolism as measured by the rate of excretion of extra-glucose in the urine following their ingestion by phlorhizinized dogs. However, this method of measuring amino-acid metabolism does not offer sufficient assurance of reliability to give any definite significance of the parallelism observed.

The refutation of Lusk's arguments does not constitute a proof that the acid products resulting from deamination of amino acids are not the cause of their specific dynamic effect. However, if there is no contradictory evidence, the simplest theory, and therefore the best theory, would seem to be that the intact amino acids are the cause of this cell stimulation.[18a]

In an attempt to decide experimentally the question whether the non-nitrogenous products of the deamination of glycine and alanine are not entirely responsible for their heating effect, results were obtained by Lusk and his coworkers which are somewhat difficult to explain. On the theory that glycollic and lactic acids are the most probable metabolic products associated with the specific dynamic effect of glycine and alanine, respectively, particular attention was given to the heating effect of these substances when administered orally to dogs. The administration of these hydroxy acids presented difficulties, since if given as such they could not be retained by experimental dogs. Using the ethyl esters, it was found [6] that ethyl glycollate was a poison. After giving ethyl lactate, and correcting the results obtained for the ethyl alcohol presumably liberated from this ester in metabolism, results were obtained indicating, though not demonstrating, that lactic acid acts as a metabolic stimulant.

In a later experiment,[19] it was found that lactic acid could be tolerated by dogs if given in a solution containing a small amount of Liebig's extract of beef, a substance shown to be without effect on heat production in the amounts used. Although the data reported were few and variable, it was concluded that lactic acid produced an increase in the heat production of dogs comparable with that produced by an equal amount of alanine. This conclusion was later rejected by Lusk in a report [20] of a number of experiments on the calorigenic action of possible acid metabolites of the amino acids, the grounds for rejection not being specified. In these experiments lactic acid was found to stimulate heat production considerably, though no comparison with alanine

[18a] "In this connection it is significant that Rapport and Katz (*Amer. J. Physiol.*, 1927, lxxx, 185) have obtained marked calorigenic effects of glycine on isolated perfused muscle. This can only be interpreted as a direct effect of the unchanged amino acid, if the intervention of the liver is essential to deamination, as the experiments of Bollman, Mann, and Magath clearly indicate (*Ibid.*, 1926, lxxviii, 258)."

[19] Atkinson, H. V., and Lusk, G., *J. Biol. Chem.*, 1919, xl, 79.

[20] Lusk, G., *J. Biol. Chem.*, 1921, xlix, 453.

was made. However, sodium lactate exerted only an inconsiderable effect on metabolism. Since lactic acid (8 grams) administered with glucose (50 grams) gave no greater heating effect than 58 grams of glucose alone, although in earlier experiments a summation of the effects of glucose and alanine was always obtained, Lusk concludes that lactic acid is probably a metabolite of glucose rather than of alanine.

Glycollic acid was found to have only an inconsiderable specific dynamic action, and sodium glycollate an even smaller effect, so that no support was produced for the belief that this acid was the cause of the specific dynamic action of glycine.

After considering these results, Lusk says: "While one may still speak of amino-acid stimulation in the sense that one speaks of the specific dynamic action of protein, one is still far removed from an actual elucidation of what transpires in the cells to induce the phenomenon."

THE ACID STIMULATION THEORY OF BENEDICT

The experiment of Lusk just reviewed was undertaken primarily to test a theory of the specific dynamic action of food, suggested by Benedict [21] as early as 1912. A citation from Benedict will best explain this theory:

"Some recent experiences with diabetics and with normal individuals under conditions of acidosis lead strongly to the belief that the presence of acid stimulates metabolism enormously. In conjunction with Dr. Joslin we have found that diabetics when under conditions of severe acidosis have a much higher metabolism than when the acidosis is mild, and indeed, although we can at present state only that the high acidosis is coincidental with the high metabolism, it is probable that the high acidosis may be the primary cause of the high metabolism. Similarly with normal individuals, when subsisting upon a carbohydrate-free diet, there is developed an acidosis which results in a very highly increased metabolism. Singularly enough, normal individuals, and indeed diabetics, indicate a tendency toward a tolerance for the presence of these acids, inasmuch as the extremely high metabolism following the rapid onset of acidosis decreases somewhat as the acidosis continues, and with diabetics it is possible to have a very high percentage of β-oxybutyric acid in the urine with a metabolism although above normal, not proportionately as much above normal as would be expected from the amount of acid present. The great significance in this connection is the fact that the rapid withdrawal of carbohydrates from diabetics who have had free diet may produce an acidosis which is so severe as to lead into coma, although the actual amount of β-oxybutyric acid present in the body and excreted in the urine is by no means as great as in longer cases of diabetics who have become more tolerant to it.

"Of particular interest is the experiment made with a severe diabetic in which after administration of 100 grams of levulose there was a noticeable increase in both carbon dioxide production and oxygen consumption, and singularly enough there was not the slightest alteration in the respiratory quotient, showing that the increased metabolism was due totally to a combustion of materials other than the ingested carbohydrates. The low respiratory quotient of 0.69 was maintained throughout the entire test, although the metabolism at times increased nearly 30

[21] Benedict, F. G., *Trans. 15th Intern. Congress on Hyg. and Demography, Washington, D. C.*, 1912, ii, 394.

per cent of that before taking the sugar. Since throughout the whole period the subject, who was an ideal one, lay absolutely quiet without an extraneous movement of any kind, it can be seen that the increase in metabolism could not be ascribed to external muscular activity.

"These experiments, therefore, lead us to the belief that there are absorbed out of the food, substances which, carried by the blood, stimulate the cells to greater metabolic activity, and at this date, 13 years later than the original statement by Friedrich Müller, all we can add is that the substances absorbed are probably of an acid nature.

"The amino acids of protein intermediary metabolism, the glycuronic and similar acids of the carbohydrate- and, indeed, protein-intermediary metabolism, and the β-oxybutyric acid of the intermediary fat metabolism, may each in proportion to the degree of formation stimulate the activity. Since experimental evidence is to the effect that the sugars react in increasing activity in the following order, dextrose, saccharose, and levulose, the asymmetric carbon atom, the ketone group, and the aldehyde group may each or all play a rôle in determining the degree of stimulation of the acid. The amount of experimental work suggested by these preliminary observations should lead ultimately to definite information regarding the molecular fragments in the foodstuffs responsible for the specific katabolic stimuli."

However, Lusk and his associates found [20, 22] that, although acids stimulate metabolism, the extent of this stimulation is not proportionate to the number of potential hydrogen ions administered nor to the reduction in CO_2-combining power of the blood, as shown in the following table:

TABLE 40. *The Effect of Acids on Heat Production as Compared with Their Effect on the CO_2 Combining Power of the Blood Plasma.*

Substance Administered	Relative No. of Potential H-Ions	Increase in Metabolism per Hr. During 2nd and 3rd Hrs.	Decrease in CO_2-Combining Power of the Plasma
		cals.	per cent
Acetic acid, 3 gms.....	1.0	3.1	0
Lactic acid, 4.8 gms.....	1.2	2.7	14
Glycollic acid, 7.6 gms.....	2.0	1.5	28
HCl, 1.8 gms.....	1.0	1.6	11

Furthermore, Chanutin and Lusk,[23] investigating the effect of the ingestion of meat, glycine, and alanine upon the alkali reserve of the organism of a dog, found that there was an increase, rather than a decrease in the CO_2-combining power of the blood plasma, continuing for five or six hours. Since it was previously shown in Lusk's laboratory [20] that the administration of sodium bicarbonate has no influence on the heat production of dogs, "it is evident that the great increase in the heat production which takes place during the hours immediately after the ingestion of meat is not determined by any change in the alkaline reserve of the blood, nor can such change be interpreted as being even a participating element in the causation of the phenomenon."

[22] Taistra, S. A., *J. Biol. Chem.*, 1921, xlix, 479.
[23] Chanutin, A., and Lusk, G., *J. Pharm. Exper. Therap.*, 1922, xix, 359.

THE AMINO-RADICAL STIMULATION THEORY OF GRAFE

In 1915, Grafe [24] reported the results of an extensive investigation of the specific dynamic action of protein. His experiments were carried out on men, dogs and rabbits, and his observations were confined to a determination of the respiratory exchange and the total N excretion in the urine. His results may be briefly summarized as follows:

1. Ammonium salts of inorganic acids administered either by mouth or subcutaneously, effected a marked increase in metabolism, though their available energy was nil.

2. Even large doses of urea, given by mouth or subcutaneously, exerted no effect on metabolism.

3. Amino acids injected subcutaneously into the diabetic organism called forth an increased metabolism, though the nitrogen-free complex is excreted in the urine as glucose.

4. All amino acids investigated, including glutamic acid, stimulated metabolism, though in different degrees. Glycine, as in most of Lusk's work, seems particularly powerful in this respect. Subcutaneous injection seemed to be more effective than oral administration.

5. Acid amides (acetamide) and amides of the amino acids (asparagin) exerted similar effects on metabolism.

A comparison of some of the results of Grafe and of Lusk, compiled by Grafe, is given in Table 41. In compiling this table Grafe excluded all of his data obtained on rabbits and all the data of the injection experiments, due to irregularities of one kind or another. The calorie figures for his own experiments are indirectly calculated from the oxygen absorption. Other exclusions were also made.

Of particular significance is the fairly constant value obtained with dogs (but not with men) when the calorie increase (presumably for the first four hours after administration) is figured to 1 gram of ingested nitrogen, and the widely varying results obtained when it is figured to 100 available calories of ingesta. On the basis of these results, Grafe concludes that with most of the amino acids and amides, their nitrogen content is closely involved in their specific dynamic action, while their calorie content bears no obvious relation to this property. He is inclined to believe, therefore, that the amino group is perhaps primarily responsible for the stimulation to metabolism, especially in view of the pronounced effect of ammonium salts.

In criticizing the compilation of Table 41 and the general conclusions of Grafe, the first point that would bear careful scrutiny is whether the arbitrary selection of the data has contributed much to the

[24] Grafe, E., *Deutsch. Arch. klin. Med.*, 1915-16, cxviii, 1.

TABLE 41. *The Results of Grafe and of Lusk as Compiled by Grafe.**

Substance	Amount Ingested in Grams	Nitrogen Ingested in Grams	Available Calories Ingested	Increase in Metabolism in Calories	Increase in Metabolism per 100 Available Calories Ingested	Increase in Metabolism per Gram N Ingested	Authority
Experiments on Dogs							
Glycine	25	4.66	52.5	20.9	39.8	4.5	Lusk
Alanine	20	3.14	71.0	12.6	18.0	4.0	"
Alanine	50[1]	7.85	177.5	35.3	19.9	4.5	Grafe
Leucine	20	2.14	118.9	6.1	5.0	2.9	Lusk
Glutamic acid	20	1.90	62.9	0.0	0.0	0.0	"
Glutamic acid	50[2]	4.75	157.3	26.4	16.8	5.6	Grafe
Tyrosine	20	1.55	109.9	7.7	7.0	5.0	Lusk
Meat	100	3.00	78.0	14.3	19.0	4.8	"
Experiments on Men							
NH_4Cl	4.13	1.09	0.0	44.2	...	40.6	Grafe
NH_4Cl	12	3.17	0.0	101.9	...	32.1	"
Glycine	50	9.35	105.0	77.3	74.0	8.3	"
Alanine	80	12.64	284.0	57.5	20.3	4.6	"
Alanine	50	7.85	177.5	58.2	32.8	7.4	"
Phenylalanine	28	2.69	174.8	55.5	31.7	20.7	"
Asparagine	47.1	8.79	96.9	78.9	81.5	8.9	"
Acetamide	42	9.95	150.5	86.0	57.1	8.6	"

* *Deutsch. Arch. klin. Med.*, 1915-16, cxviii, 22, 25.
[1] A small part was vomited.
[2] Given as the sodium salt.

constancy of the calculation of increased metabolism per gram of ingested nitrogen. This study seems particularly important in view of the variable results that Lusk often secured for the same amino acid.

In commenting upon Grafe's experiments, Atkinson and Lusk[7] note that some of the results confirm and others contradict those of Lusk while others are self-contradictory:

> For example, Grafe found in a dog that the absorption of oxygen was increased between 16 and 17 per cent during 3⅓ hours following the ingestion of 50 grams of glutamic acid (as a sodium salt), whereas Lusk had found no increase after giving 20 grams. Furthermore, Grafe reported that after giving 67.5 grams of asparagine to a man the metabolism was uninfluenced during successive periods, respectively, of 4.5, 4 and 2 hours duration, although he found an increase of 11 per cent in the oxygen absorption after giving 47 grams to another man. This last experiment is open to the following criticisms: (*a*) The man was in the hospital undergoing treatment for syphilis. (*b*) The basal metabolism was determined 5 days before the day of asparagine ingestion, during which interval the patient is stated to have increased in weight from 50.2 to 51.1 kilos. (*c*) The respiratory quotient of the 10 hour interval of the basal metabolism period was 0.95, notwithstanding the fact that this period began 12 hours after taking food. Such a respiratory quotient could scarcely have been obtained had the patient been given sugar in large excess on the day previous.[25] This leads to

[25] Benedict, F. G., Emmes, L. E., and Riche, J. A., *Amer. J. Physiol.*, 1910-11, xxvii, 383.

the suspicion that the oxygen determination was too low. The carbon dioxide excretion rose only 5 per cent as a result of the asparagine ingestion.

This questionable determination of the basal metabolism throws doubt upon reported increases in the metabolism of this man, of 13 per cent after giving 28 grams of phenylalanine; of 14 per cent after giving 12 grams of ammonium chloride; and of 22 per cent after giving 42 grams of acetamide.

The particular instances of contradiction between Grafe's results and those of Lusk's laboratory refer to glutamic acid and asparagine, which were found to be without effect, and to acetamide, which Atkinson and Lusk found was not deaminized in the dog and was without effect on heat production. On the basis of their own results, the latter investigators conclude:

It is evident, therefore, that asparagine and glycocoll, which contain the same percentage quantity of nitrogen, behave very differently in metabolism, the first being without specific dynamic action and the other exerting the most powerful specific dynamic action of any of the amino acids contained in protein which have thus far been tested. Therefore, the specific dynamic action of protein is not due to the NH_2 radicle of the amino acid molecule.

THE RELATION OF THE ENDOCRINE GLANDS TO THE SPECIFIC DYNAMIC EFFECT OF FOOD

A new viewpoint on the cause of the specific dynamic action of protein has resulted from observations upon subjects suffering from endocrine deficiencies. The investigations of Plaut,[26] Rolly,[27] Bernhardt,[28] and Liebesny [29] indicate that hypophyseal (pituitary) disorders are frequently associated with diminished specific dynamic action of protein. Liebesny's experiments also show that a pathological condition of the autonomic nervous system, as in certain neuroses, Raynaud's disease, and scleroderma may have the same effect. Wang and Strouse,[30] in respiration experiments on twelve obese subjects, found markedly subnormal specific dynamic effects of protein, carbohydrate and fat. However, no clinical description of these subjects is given and the results, therefore, cannot be referred to any particular glandular deficiency. Similar results are reported by Jaquet and Svenson,[31] though in the absence of normal standards of comparison, their significance is not so evident. Castration or hypofunctioning of the sex glands does not apparently affect the specific dynamic reaction to protein.[29]

The evidence with reference to the relation of the thyroid to the specific dynamic action of protein is mainly negative. Investigations of patients with Graves' disease, and myxedema, in which the thyroid is

[26] Plaut, R., *Deutsch. Arch. klin. Med.*, 1922, cxxxix, 285; *ibid.*, 1923, cxlii, 266. Plaut and Schadow (*Deutsch. Arch. klin. Med.*, 1925, cxlviii, 214) have found in certain cases of pathologically reduced specific dynamic effect of protein, a subnormal aminoacidemia also.
[27] Rolly, F., *Deutsch. med. Wochnschr.*, 1921, xlvii, 887, 917.
[28] Bernhardt, H., *Z. klin. Med.*, 1924, xcix, 149.
[29] Liebesny, P., *Biochem. Z.*, 1924, cxliv, 308.
[30] Wang, C. C., and Strouse, S., *Arch. Int. Med.*, 1924, xxxiv, 573.
[31] Jaquet, A., and Svenson, N., *Z. klin. Med.*, 1900, xli, 375.

over- or underfunctioning, respectively, indicate a specific dynamic action of food or of protein that is within normal limits.[32, 33, 26, 27, 29] Liebesny's work is particularly convincing on this point.

However, the specific dynamic action of carbohydrates seems to be closely dependent upon thyroid function. Thus, Eckstein and Grafe [34] reported experiments indicating that extirpation of the thyroid in dogs led to a diminution of specific dynamic effect of a low-nitrogen ration, and, more recently, Baumann and Hunt [35] showed that complete thyroidectomy in rabbits led to a gradual disappearance of the specific dynamic reaction to glucose. This disappearance of specific dynamic action was complete in about 65 days, approximately the life of thyroxin in the body according to Boothby and Sandford,[36] and could be restored upon thyroxin feeding. Incomplete thyroidectomy resulted in a lowered specific dynamic effect. It is to be hoped that these excellent experiments will be repeated using protein instead of glucose. Possibly the specific dynamic effect of protein or amino acids may be differentiated from that of glucose with respect to thyroid involvement as well as in the respects already noted.*

DOES THE AMINO-ACID MAKE-UP OF PROTEINS DETERMINE THEIR SPECIFIC DYNAMIC EFFECT?

The investigations of Lusk with reference to the power of different amino acids to stimulate the heat production of the dog, led him to the conclusion that the specific dynamic effect of proteins was in all probability due mainly to their content of the two simplest amino acids, glycine and alanine. One would expect, therefore, that proteins would differ in specific dynamic action in accordance with their percentage content of these two acids. Rapport,[37] working in Lusk's laboratory,

[32] DuBois, E. F., *Arch. Int. Med.*, 1916, xvii, 915.

[33] Aub, J. C., and Means, J. H., *Arch. Int. Med.*, 1921, xxviii, 173.

[34] Eckstein, E., and Grafe, E., *Z. physiol. Chem.*, 1919, cvii, 73. Miyazaki and Abelin (*Biochem. Z.*, 1924 cxlix, 109) have noted large increases in the specific dynamic effects of carbohydrates and fats following the administration of thyroid material, while Abelin (*ibid.*, 1923, cxxxvii, 273), has noted the same phenomenon with reference to proteins. *See also* Abelin, J., *Biochem. Z.*, 1924, cliv, 52.

[35] Baumann E. J., and Hunt, L., *J. Biol. Chem.*, 1925, lxiv, 709.

[36] Boothby, W. M., and Sandiford, I., *J. Biol. Chem.*, 1924, lix, p. xl.

* Aub, Everett and Fine (*Amer. J. Physiol.*, 1926-7, lxxix, 559) have obtained some very interesting results on cats indicative of a relation between the nervous system and the specific dynamic effect of amino acids. The intravenous injection of glycine, but not of glutamic acid, produces a definite specific dynamic effect in the decerebrate cat. However, no increase in metabolism occurs when glycine is injected intravenously into cats anesthetized by urethane or paraldehyde.

Liebeschütz-Plaut and Schadow (*Arch. ges. Physiol.*, 1926, cxiv, 537) have recently reported a failure to obtain any considerable calorigenic effect of the intravenous administration of amino acids into dogs, although intraduodenal administration exerted the usual effect. This disagreement between the two methods of administration is urged by the authors against Lusk's ideas of the cause of the specific dynamic effect of amino acids. The failure of effect after intravenous injection however, may be contrasted with the positive results secured by Aub, Everett and Fine, and by Wilhelmj and Bollman (see footnote to page 439) and also with those of Grafe [24] after subcutaneous administration. Krzywanek (*Biochem. Z.*, 1923, cxxxiv, 500) also, has experienced no trouble in securing increases in the heat production of dogs following parenteral introduction of amino acids.

[37] Rapport, D., *J. Biol. Chem.*, 1924, lx, 497.

has recently undertaken to test this inference by determining the specific dynamic action of several proteins differing in amino acid composition. For this purpose he used gelatin, containing approximately 26 per cent glycine and 9 per cent alanine, beef, containing approximately 4 per cent glycine and 8 per cent alanine, gliadin, containing no glycine and about 2.0 per cent alanine, and casein, also containing no glycine and only about 1.5 per cent alanine. One would expect from Lusk's work that the specific dynamic effect of these proteins would decrease in the order given, but Rapport was not able to distinguish between the four proteins in their effects upon the heat production of the dog. As an average of a four-hour period starting with the second hour after the protein meal, gelatin gave an increase in heat production of 29 per cent over the basal in two experiments, beef an average increase in three experiments of 34 per cent, gliadin an average increase in two experiments of 35 per cent, and casein an average increase in two experiments of 31 per cent. Codfish containing no glycine and a questionable trace of alanine nevertheless gave an average increase in heat production of 35 per cent over the basal in one experiment, while chicken, containing about 0.68 per cent glycine and 2.28 per cent alanine, increased metabolism an average of 31 per cent in one experiment.

In explaining the absence of any apparent relation between the amino acid make-up of a protein and its specific dynamic effect on metabolism, it is suggested that in the course of the metabolism of different proteins there is a constant and equal synthetic production from them of the two amino acids, glycine and alanine, exerting the most marked heating effect. However, Csonka [38] showed that when a pig is given benzoic acid continuously, thus providing a constant and maximum stimulus to glycine synthesis, the quantity of glycine synthesized from casein and excreted in the urine as hippuric acid, is far less than the amount produced and eliminated after the administration of gelatin.

Weiss and Rapport [39] conducted further investigations in an attempt to elucidate the anomalous results of Rapport. When casein, which contains no glycine, was administered with glycine to a dog, there was no greater specific dynamic effect than when casein was given alone. However, gelatin, a protein rich in glycine, also gave as great a specific dynamic effect when fed alone as when fed with glycine (10 grams). A partial summation of effect resulted when the amount of gelatin given the dog was reduced from 39 to 10 grams, or when the amount

[38] Csonka, F. A., *J. Biol. Chem.*, 1924, lx, 545.
[39] Weiss, R., and Rapport, D., *J. Biol. Chem.*, 1924, lx, 513. *See also* Rapport, D., and Beard, H. H., *Proc. Amer. Physiol. Soc., Am. J. Physiol.*, 1927, lxxxi, 505.

of glycine given with 39 grams of gelatin was increased from 10 to 20 grams. Similar results were obtained with alanine additions to casein or gelatin. The limited conditions necessary in obtaining a distinct summation of the specific dynamic effects of protein and amino acid are in contrast to the summation, complete or partial, of the specific dynamic effects of (*a*) two portions of the same amino acid or of the same protein, or (*b*) two portions of different amino acids or of different proteins, under a wide range of conditions. Weiss and Rapport conclude from these facts that the specific dynamic effect of glycine or of alanine is neutralized in whole or in part when these amino acids are given to a dog in conjunction with protein. However, it seems just as reasonable to conclude that the protein effect is neutralized by the amino acid, or that the neutralization of effect is mutual.

Whether this neutralization occurred in the gastro-intestinal tract, or in the blood or tissues, was investigated by administering the amino acid subcutaneously or intravenously at the same time that gelatin was given by mouth. Again the specific dynamic effect was no greater than for gelatin alone, indicating that the inhibition of the specific dynamic effect of glycine by protein, or of protein by glycine, as the case may be, may occur in the blood or tissues. In this connection it was shown that the specific dynamic effect of glycine is not appreciably different whether it be given by mouth, subcutaneously, or intravenously. It was further shown that the specific dynamic effect of glycine is not inhibited by the simultaneous administration of asparagine, an amino acid amide having itself no specific dynamic effect in the quantities used.

THE SUMMATION OF SPECIFIC DYNAMIC EFFECTS

A question of considerable practical importance relates to the summation of specific dynamic effects. Lusk's investigations were concerned to a large extent with this question, though unfortunately the results are not capable of a simple interpretation. In one of the first reports concerned with the summation of specific dynamic effects,[40] it was shown that the meat in the standard diet fed the dogs could be reduced in quantity from 100 to 33 grams daily with no effect on its specific dynamic action. The addition of amino acids to the standard diet produced no increase in heating effect unless the extra heat induced by the standard diet was less than the extra heat induced by the amino acid alone. It was concluded, therefore, that the metabolism of plethora following fat and carbohydrate ingestion, and the metabolism of amino acid stimulation, cannot be added to each other; there is no summation of effect when

[40] Lusk, G., *J. Biol. Chem.*, 1912, xiii, 185.

both influences are brought into effect simultaneously. Aside from its experimental basis, this conclusion seems reasonable, since the situation is quite analogous to that of the effect of sugar ingestion by a working dog.[11] The stimulus of muscular activity, by preventing a plethora of glucose molecules in the tissues, inhibited the metabolism of plethora, and no summation of effects was observed.

However, in a later publication,[6] experiments of different import were reported. After giving 50 grams of glucose with 20 grams of glycine, the increase in metabolism was almost as great as the sum of the increases induced when each substance was given alone. Alanine 20 grams and glucose 50 grams also exhibited a marked summation, though not mathematically complete. Lusk stated, therefore, that these results nullified his former opinion, though obviously the preceding experimental data are as valid as when first obtained, and have since been explained in the light of the results of Weiss and Rapport.[39, p. 541] A similar summation of the effect of fat and glucose, and of fat, glucose, and glycine was reported in a later publication,[41] while later still [20] a more definite statement of Lusk's opinion on this matter is given, *i.e.*:

> "One of the peculiarities of the specific dynamic action of amino acids, glucose, and fat is that the extra heat produced by one [each?] of the three is specific and that when two or more of the materials are given together the extra heat production is the sum of the influences which each substance given alone would have induced. In line with this knowledge it follows that if glucose and lactic acid be given together and lactic acid behaves like a metabolite of alanine, the heat production will be greater than when glucose alone is given, but if it behaves like a metabolite of glucose no appreciable rise will be observed."

Since glucose 50 grams and lactic acid 8 grams gave no greater specific dynamic effect than glucose 58 grams, it is concluded that lactic acid behaves like a metabolite of glucose rather than of alanine. On the other hand, glucose 50 grams + acetic acid 3 grams produced an increase in heat production only slightly less than the sum of the increases of glucose and acetic acid administered separately. Hence, acetic acid does not behave like a glucose metabolite, but more like a product of the beta-oxidation of a fatty acid.

The interpretation thus placed upon previous experimental data with reference to the summation of specific dynamic effects, and the use to which this interpretation has been put, implies that the relation between the amount of any given nutrient administered and the resulting increase in heat production is not particularly close, although even small additions of another nutrient will always give an increase in specific dynamic effect. The first implication may be abundantly illustrated from

[41] Murlin, J. R., and Lusk, G., *J. Biol. Chem.*, 1915, xxii, 15.

Lusk's published results, but the second implication appears to be not securely founded upon experimental observation.

In evaluating Lusk's experimental results bearing upon the summation of specific dynamic effects, it should be kept in mind that they refer to the "peak" effect on metabolism, or the most intense effect of nutrients, or at most, to a period of 2 to 4 hours' duration including the peak effect. In other words, he was concerned with the question: Are the maximum increases in metabolism induced by two different nutrients when fed separately added together when the nutrients are fed together? The answer to the question is of interest more from the academic than from the practical standpoint. A more practical problem relates to the *total* heating effect of different nutrients and foods on metabolism, because evidently as this total effect varies the value of the nutrient or food as a source of energy to the animal organism will vary in an inverse fashion.

In order to determine the total specific dynamic effect of a food or nutrient, the experiment should start with a determination of the basal metabolism and should continue until this initial level of metabolism is again attained. No experiments of this description with reference to protein feeding or amino acid feeding appear in the literature, although many of the experiments of Benedict and Carpenter [42] approximated this ideal. These investigators found that meals predominantly protein in character had a varied effect on the basal metabolism of human subjects. Expressing the total extra-calories produced above the basal level during the periods of observation as a percentage of the physiological fuel value of the food consumed (computed generally from Rubner's factors), they obtained specific dynamic effects ranging from 2 to 33, and averaging approximately 11. These figures must be considered, however, as minimal, since in many experiments it is probable that the entire increment in heat production was not measured. The average of 11 for protein and meals rich in protein is to be compared with an average of 6 for sugars and foods predominantly carbohydrate, and an average of only 2 per cent for foods rich in fat.

This percentage of the fuel value of the diet ingested appearing as extra heat is called the "cost of digestion" by Benedict and Carpenter, a term not committing its sponsors to any theory concerning the cause of the increased metabolism.

THE SECONDARY SPECIFIC DYNAMIC EFFECT OF PROTEIN

Thus far, the immediate effect only of the ingestion of protein on metabolism has been considered. However, the level of protein nutrition

[42] Benedict, F. G., and Carpenter, T. M., *Carnegie Inst. Publ.* 261, 1918.

of an animal exerts also a marked effect upon its post-absorptive metabolism, probably by determining the amount of the so-called "circulating protein" in its tissues, the protein that is gradually lost on lowering the level of protein intake with no detrimental effect on the animal. This is sometimes called "deposit protein," but whether it is protein at all is questionable. The proof of its existence rests entirely upon the results of nitrogen balance studies; but the fact that the excessive feeding of zein, an incomplete protein, leads to a prolonged lag in the adjustment of the urinary nitrogen to the endogenous level in a subsequent period of starch feeding,[43] might be taken to indicate that the temporary retention of nitrogen on high protein levels is not necessarily due to a retention of protein.

However this may be, it was shown by Rubner,[3, p. 246] that the ingestion of excessive amounts of protein by a dog for several days occasions a gradual rise in total heat production until nitrogen equilibrium is attained. Similarly, Atkinson, Rapport, and Lusk [44] noted that the basal metabolism of a dog on their standard mixed diet, amounting to 16 calories per hour, was increased after 8 days of meat feeding to 19.7 calories per hour on the second day following a return to the standard diet. Subsequent daily determinations on this diet gave values of 18.3, 17.3, 18.2 and 17.6 calories. Even after two and one-half weeks the basal metabolism was 17.1 calories per hour, showing a persistently higher level than obtained before the meat feeding. The authors generalize on the significance of "deposit protein" and "improvement protein," as follows:

> "The bodily condition is always a factor to be considered in the determination of basal metabolism. The condition of muscular strength, accompanied by the addition of an improvement quota of protein [45] to the cells of the body, results also in a higher metabolism in the dog [6] and in man.[46] It is possible that a lesser amount of deposit protein and of improvement protein may be in part responsible for the lower basal metabolism of women, first pointed out by Gephart and DuBois.[47] Thus, the recent experiments of Blunt and Bauer [48] show that undernutrition does not play a large part in reducing the metabolism of women."

In the same connection it is pointed out that Benedict, Miles, Roth, and Smith [49] were the first to associate the loss of "deposit protein"—called by them "surplus cellular nitrogen"—with the reduction in the basal metabolism of men consequent upon a marked reduction in caloric intake.

[43] McCollum, E. V., and Steenbock, H., *Wisconsin Agr. Exp. Sta. Res. Bull.* 21, 1912, p. 69.
[44] Atkinson, H. V., Rapport, D., and Lusk, G., *J. Biol. Chem.*, 1922, liii, 155.
[45] Rubner, M., *Arch. Physiol.*, 1911, 76.
[46] Benedict, F. G., and Smith, H. M., *J. Biol. Chem.*, 1915, xx, 243.
[47] Gephart, F. C., and DuBois, E. F., *Arch. Int. Med.*, 1916, xvii, 902.
[48] Blunt, K., and Bauer, V., *J. Home Econ.*, 1922, xiv, 171.
[49] Benedict, F. G., Miles, W. R., Roth, P., and Smith, H. M., *Carnegie Inst. Washington, Publ.* 280, 1919.

Such experiments as those cited indicate that the basal metabolism is a good index to the "vitality" of an animal, and suggest in turn that the protein make-up of the tissues and circulating fluids of the body, or their content in protein derivatives, may be intimately involved in their physiological efficiency.

CHAPTER IX

THE ENDOGENOUS CATABOLISM.

EARLY THEORIES OF PROTEIN METABOLISM

The protein, or amino acid, catabolism that has thus far been considered relates to the amino acids coming to the tissues from the alimentary tract. There is, however, a different type of protein catabolism, one that relates to the disintegration of the tissues themselves. Until the classical work of Folin, a good deal of confusion existed in the literature as to the relation existing between these two different kinds of catabolism. At that time, two different theories, each proposed by a prominent German physiological chemist, were contending for general recognition. Folin briefly describes these theories as follows:[1]

> The theory of Pflüger is essentially a modification of an earlier one advanced by Liebig, and is very old. The theory of Voit was first formulated in 1867 after the original theory of Liebig had become untenable, and for a long time Voit's theory enjoyed almost universal acceptance, although it, too, had to be modified in order to be consistent with the facts brought out by Pflüger. Since 1893 Voit's theory may be said to have lost ground. In that year Pflüger published an exceedingly searching criticism of nearly all the facts which Voit had advanced in favor of his theory, and showed that they were either erroneous or capable of a different interpretation. This criticism, accompanied as it was by the experiments of Schöndorff, has never been refuted.
>
> The two theories are briefly as follows: According to Voit the protein of the absorbed food passes through the blood to the different tissues and cells, and is there catabolized under the influence of the living protoplasm, but without first becoming an integral part of the latter. Voit's fundamental conception seems to me to be that the living protoplasm is in a state of suspension, the circulating protein is in solution, and the chemical decompositions that constitute protein catabolism take place only in solution. The small amount of living protoplasm which dies in the course of twenty-four hours is at first only dissolved, thereby becoming a part of the circulating protein derived directly from the food.
>
> Pflüger, on the other hand, believes that there is a very decided chemical difference between circulating protein and living protoplasm. The former is comparatively stable toward oxidizing reagents, while the latter is in a very unstable equilibrium and is particularly susceptible to oxidation. All the protein catabolized is first transformed into bioplasm, becomes an integral part of the living tissue, and only as such undergoes the oxidation that is supposed to constitute the most fundamental chemical decomposition of protein catabolism.

Schöndorff's experiments, above referred to as supporting Pflüger's conceptions rather than those of Voit, taken at their face value simply indicate "that it is the state of nutrition in muscle cells and not the circulating protein which is the determining factor in the protein catab-

[1] Folin, O., *Amer. J. Physiol.*, 1905, xiii, 117.

olism." But this conclusion bears a rather dubious connection with the point at issue, since the circulating protein might be readily catabolizable and yet the amount catabolized per unit of time might be determined by the condition of the tissues. This is but an illustration of the difficulty of bringing experimental methods to bear upon such an abstruse problem as that with which these two theories are concerned. Which constituents of the tissues are living and which are dead would seem to be a purely philosophical question, that may be productive of much argument, but that is almost inaccessible to experimental inquiry. A point of great importance in the evaluation of Folin's theory is that it is the outgrowth of experimentally determined facts. It is a reasonable interpretation of established laws relating to the factors governing the chemical composition of urine.[2] These laws relate to the changing composition of urine with changes in the level of protein intake; their existence was not suspected by either Voit or Pflüger, and, as a consequence, the theories of these investigators are either incompatible with the facts established by Folin, or they provide no adequate explanation of them.

FOLIN'S THEORY OF PROTEIN METABOLISM

Folin's theory is best explained in his own words:

> We have seen . . . that the composition of urine, representing 15 grams of nitrogen, or about 95 grams of protein, differs very widely from the composition of urine representing only 3 grams or 4 grams of nitrogen, and that there is a gradual and regular transition from the one to the other. To explain such changes in the composition of the urine on the basis of protein catabolism, we are forced, it seems to me, to assume that that catabolism is not all of one kind. There must be at least two kinds. Moreover, from the nature of the changes in the distribution of the urinary constituents, it can be affirmed, I think, that the two forms of protein catabolism are essentially independent and quite different. One kind is extremely variable in quantity, the other tends to remain constant. The one kind yields chiefly urea and inorganic sulphates, no creatinin, and probably no neutral sulphur. The other, the constant catabolism, is largely represented by creatinin and neutral sulphur, and to a less extent by uric acid and ethereal sulphates. The more the total catabolism is reduced, the more prominent become these representatives of the constant catabolism, the less prominent become the two chief representatives of the variable catabolism.
>
> The fact that the urea and inorganic sulphates represent chiefly the variable catabolism does of course not preclude the possibility that they also represent to some extent the constant catabolism; but I have reason to believe that it is possible to plan feeding experiments which will yield urines containing very much smaller per cents of these two constituents than I have yet obtained. We know from the experiments of Sivén that it is possible to reduce the total protein catabolism still more, and I am confident that in such cases the per cent of urea-nitrogen will sink still lower, and that the nitrogen of the other constituents, particularly of the creatinin, will again show a corresponding increase.
>
> If there are two distinct forms of protein metabolism represented by two different sets of waste products, it becomes an exceedingly interesting and important problem to determine, if possible, the nature and significance of each. The fact that the creatinin elimination is not diminished when practically no protein is furnished with the food, and that the elimination of some of the other con-

[2] Folin, O., *Amer. J. Physiol.*, 1905, xiii, 66.

stituents is only a little reduced under such conditions, shows why a certain amount of protein must be furnished with the food if nitrogen equilibrium is to be maintained. It is clear that the metabolic processes resulting in the end-products which tend to be constant in quantity appear to be indispensable for the continuation of life; or, to be more definite, those metabolic processes probably constitute an essential part of the activity which distinguishes living cells from dead ones. I would therefore call the protein metabolism which tends to be constant, tissue metabolism or endogenous metabolism, and the other, the variable protein metabolism, I would call the exogenous or intermediate metabolism.

To Folin, the difference in the character of the end-products of these two types of protein metabolism signified not only a difference in their catabolic processes, but also a difference in the tissues in which they occurred. The endogenous metabolism appeared to be related to the oxidative processes occurring in all tissues of the body, while the exogenous metabolism, insofar as it related to the nitrogenous end-products, mainly involved hydrolysis, and was probably localized in special tissues, such as the liver. It is to be noted, however, that the assumption of a localized formation of urea, diametrically opposed to Folin's later views, was based largely upon inadequate information concerning the occurrence of urea in animal tissues, and particularly upon its supposed absence in muscle or its presence in only infinitesimal traces.

THE RELATION BETWEEN FOLIN'S THEORY AND PROTEIN REQUIREMENTS

Folin's theory of an endogenous protein metabolism, distinct from and independent of the exogenous metabolism, has been widely accepted by biochemists throughout the world, at least until recently. It is in close agreement with Rubner's [3] well-known classification of protein requirements, *i.e.*:

1. The "repair quota," required to replace the protein lost in the "wear-and-tear" quota of the protein metabolism.
2. The "growth quota," required to provide protein for growth.
3. An "improvement quota," required by the adult organism for the recuperation of protoplasmic tissue after fasting or during convalescence from wasting diseases.
4. The "dynamic quota," including protein consumed in excess of requirements. Such an excess is deaminized and used as a source of energy, either immediately or ultimately.

In this scheme, the "repair quota" would be related to the endogenous metabolism of Folin, and the "dynamic quota" would be related to the catabolic phase of the exogenous protein metabolism. The "growth quota" and the "improvement quota" would be represented by the

[3] Rubner, M., *Arch. Hyg.*, 1908, lxvi, 45.

anabolic phase of the exogenous protein metabolism, and would be measurable by the nitrogen balance.

This classification has been extended by Ringer,[4] on the basis of his own results and those of Landergren, to include a quota of protein necessary above the "wear-and-tear quota" in the absence of carbohydrate from the diet for the maintenance of the sugar content of the blood. This quota can be spared by carbohydrate but apparently not by fat, since a physiological transformation of fat into glucose has never been proven. Convincing confirmation of the actuality of this quota is offered by Ringer. Phlorhizin diabetes is characterized by a condition of hypoglycemia and a greatly increased catabolism of protein. The administration of glucose to phlorhizinized animals, even though it is not utilized, will lower the protein catabolism markedly. On the other hand, in pancreatic diabetes, characterized by hyperglycemia and a smaller increase in protein catabolism, the administration of glucose has no effect on the excretion of urinary nitrogen. Nash [5] has recently shown that the injection of insulin into a phlorhizinized dog will also markedly lower the protein catabolism as measured by the excretion of urinary nitrogen.

"DEPOSIT PROTEIN" AND ITS SIGNIFICANCE

In Folin's experiments and in experiments of like nature in which subjects are removed from normal rations to rations containing little or no nitrogen, the urinary nitrogen does not immediately adjust itself to the endogenous level. It may be several days—or even weeks—before this level is attained, the length of the transition period depending upon the protein level of the diet or ration preceding the period of specific nitrogen hunger. An illustration may be taken from some experiments by Thomas.[6] After the removal of nitrogenous constituents from the diet, the daily collections of urine in one experiment contained, respectively, 13.70 grams, 7.30 grams, 5.70 grams, 3.40 grams, and 3.04 grams of nitrogen. On returning to a nitrogen-containing dietary the urinary excretion again lags behind the intake of nitrogen, possibly for several days. The picture presented is that of a reservoir of nitrogenous material, the level of which is closely related, in a direct fashion, with the level of protein feeding.

Considerable speculation has been aroused concerning the significance of this reservoir. By Rubner, Thomas, Lusk and various other writers, it is referred to as "storage protein" or "deposit protein," and is con-

[4] Ringer, A. I., *J. Biol. Chem.*, 1912, xii, 431.
[5] Nash, T. P., *J. Biol. Chem.*, 1923, lviii, 453.
[6] Thomas, K., *Arch. Physiol., Suppl. Bd.*, 1910, 249.

ceived of as a temporary storage of protein in the blood or cellular fluids, related possibly to the vitality of the animal as reflected, for example, in its basal metabolism.[7] However, experimental foundation for this viewpoint is lacking, and it is not in harmony with an experiment reported by McCollum and Steenbock [8] in which the urinary excretion of nitrogen by a pig was shown to exhibit a prolonged lag after changing from a diet containing zein to one of starch and salts. On the first day of the starch diet, the urine contained 8.62 grams of nitrogen, and on succeeding days 6.04 grams, 4.78 grams, 3.70 grams, 2.34 grams, 2.25 grams, 1.45 grams, 1.45 grams, and 1.26 grams. The period of zein feeding lasted 17 days, and was preceded by a starch-feeding period of 11 days, during which the daily excretion of urinary nitrogen was reduced to 104 grams. In this experiment, therefore, the "reservoir" under discussion was filled by the feeding of an incomplete protein, which could not induce protein synthesis in the animal body.

This experiment of McCollum and Steenbock would favor the view that the temporary storage of nitrogenous material in accordance with the level of protein feeding must be in the form of non-protein material. This is in agreement with the experimental results (and their interpretation of them) of Folin and Denis,[9] who believe that the reserve materials are amino acids, and that they are retained largely in the muscles. According to these authors:

"The existence of such a reservoir must be taken into account in our theories of protein metabolism, for it certainly ought to make at least some points clear which were not clear before. The peculiar lag extending over several days in the establishment of a constant level of nitrogen elimination when extreme changes are made in the nitrogen intake is probably due to a filling or a depletion as the case may be of the reservoir."

The question might be settled more definitely by a comparison of the composition of the tissues of animals on a high level of protein feeding with that of the tissues of animals in a condition of specific nitrogen hunger. Apparently the only attempt in this direction has been reported by Mitchell, Nevens and Kendall [10] on the rat. In these experiments the amino nitrogen content of the protein-fed and of the protein-starved rats was remarkably constant, though the creatine content of the latter rats was distinctly lower than that of the former, and the urea content also appeared to be lower. Probably the rat is not a suitable subject in settling

[7] Atkinson, H. V., Rapport, D., and Lusk, G., *J. Biol. Chem.*, 1922, liii, 162.
[8] McCollum, E. V., and Steenbock, H., *Wisc. Agr. Exp. Sta., Res. Bull.* No. 21, 1912, p. 69.
[9] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 94.
[10] Mitchell, H. H., Nevens, W. B., and Kendall, F. E., *J. Biol. Chem.*, 1922, lii, 417.

this particular point, since its rapid adjustment to changes in the level of protein feeding, demonstrated in these experiments, indicate only a limited capacity for the storage of "reserve" nitrogenous material.*

FACTS AND THEORIES OPPOSED TO THE THEORY OF FOLIN

While Folin's conception of the endogenous protein metabolism has been generally accepted, it has not been consistent with many of the recent experimental results relative to protein utilization. For example, gliadin will cover the maintenance requirement for protein indefinitely, according to the investigations of Osborne and Mendel, but it will not support any considerable growth. However, if the endogenous protein metabolism represents protein destruction, it is difficult to conceive how the protein thus destroyed can be replaced except by proteins capable of providing completely for the requirements of protein synthesis. The situation becomes more involved when it is shown [8] that such incomplete proteins as zein and gelatin will partially replace the nitrogen losses of the endogenous metabolism, and that even ammonium salts [11] may apparently be used to some extent for this purpose.

McCollum [8] has attempted to reconcile some of these facts with Folin's theory by assuming that "the processes of cellular catabolism and repair do not involve the destruction and resynthesis of an entire protein molecule." The somewhat clearer statement of the same conclusion by Steenbock and Gross [12] is as follows: "In endogenous protein catabolism the destruction of all amino acids is not in proportion to the extent in which the individual amino acids occur in the disintegrating protein molecule." However, it is difficult to conceive of the tissue proteins undergoing only partial hydrolysis, always retaining some of the amino acids, such as lysine, in the intact portion. There are no known regulating agencies in the tissues to which such a conservation of amino acids can reasonably be ascribed. Tissue proteases are not known to possess any such specificity of action.

Other attempts to account for the utilization of incomplete proteins and simple nitrogenous compounds in replacing endogenous losses amount to a complete repudiation of Folin's idea that there is a basal catabolism of protein, commensurate with the protein requirement for the maintenance of life. Reference may be made to the explanations of Sherman [13] and of Osborne and Mendel.[14]

* A quantitative study of the amount of "deposit protein" in the human organism, as measured by the rate of nitrogen elimination during a 30-day period on a nitrogen-free diet, has been reported by Deuel, Sandiford, Sandiford and Boothby (*J. Biol. Chem.*, 1928, lxxvi, 391).

[11] Albertoni, P., *Ergeb. Physiol.*, 1921, xix, 594. *See also* Scharrer, K., and Strobel, A., *Z. angew. Chem.*, 1925, xxxviii, 601.

[12] Steenbock, H., and Gross, E. G. *J. Biol. Chem.*, 1918, xxxvi, 285.

[13] Sherman, H. C., *J. Biol. Chem.*, 1920, xli, 97.

[14] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1914, xvii, 328.

Sherman's theory was advanced primarily to explain the fact that the nitrogen of mixtures of amino acids, so incomplete as not to cover the maintenance requirements, may nevertheless be utilized, often to a large extent, while even single nitrogenous compounds, such as individual amino acids or some of the ammonium salts may have a favorable influence on the nitrogen balance of animals. In accounting for these facts, the conception of the reversible reaction has been put forward in relation to the hydrolysis of tissue proteins and the deamination of the amino acids thus formed. It is supposed that the hydrolysis of tissue proteins, constituting the initial step in the endogenous catabolism, may be checked or even reversed by an increase in the concentration of amino acids in the tissues during digestion and absorption. Thus, any amino acid will function in the maintenance of body protein, though the more complete the mixture of amino acids coming from the intestinal tract the more marked will this effect be, since each amino acid could be expected to check the hydrolysis only at the point at which that particular amino acid would be liberated from the protein molecule. According to this theory the utilization of the nitrogen of incomplete mixtures of amino acids is only apparent. The real effect is simply an inhibition or retardation of the endogenous catabolism. In a similar way, it is assumed that the apparent utilization of ammonium salts is in fact only a retardation of another reversible reaction, in this case that of the deamination of amino acids.

The theory of Osborne and Mendel of the endogenous metabolism was advanced specifically to explain why certain proteins, notably the gliadin of wheat, may supply the nitrogenous needs of an animal in maintenance, and yet be entirely inadequate for the purposes of growth. It was their idea that the maintenance protein requirement may in reality be a requirement for definite amino acids that may serve special physiological functions; for example, serving as the material for the synthesis of the active constituents of the secretions of the endocrine glands. The body protein, on a nitrogen-free diet, would thus undergo degradation only because by this method some essential amino acids are liberated. The theory is consistent with the view that the endogenous catabolism would be greater on a non-nitrogenous diet than on a nitrogenous one, since in the latter case the nitrogenous requirements for hormone precursors would be covered by food protein, not body protein; hence the hydrolysis of body protein would cease, and the endogenous catabolism would be reduced to the degradation of the hormones themselves in the course of metabolism. The endogenous nitrogen would hence be reduced on a diet containing complete proteins, possibly to only a small fraction of its value on a nitrogen-free diet.

If the theory of Sherman as to the nature of the endogenous catabolism is correct, or if the views of Osborne and Mendel conform with the facts, then no constant basal catabolism of body tissue exists. According to these theories, the endogenous catabolism would be largely or entirely suppressed when an animal is receiving protein in its food, either because of a retardation of the hydrolysis of tissue protein caused by the mass action of the amino acids coming from the digestive tract, or because of the complete suppression of the hydrolysis of tissue protein, initiated, in the absence of amino acids of dietary origin, by the urgent need of the body for certain nitrogenous hormones; with the influx of amino acids from the intestinal tract, exogenous precursors of these hormones become available, and the endogenous catabolism is reduced to the inevitable destruction of the hormones themselves in the course of general metabolism.

THE INDEPENDENCE OF THE ENDOGENOUS AND THE EXOGENOUS METABOLISM

The constancy of the endogenous catabolism, assumed by Folin, is difficult to prove or disprove experimentally. Its justification rests upon the constant output of creatinine and neutral sulfur, the characteristic end-products of this type of catabolism. However, creatinine contains considerably less than half of the total urinary nitrogen excreted on a nitrogen-free diet, so that the constancy of creatinine excretion in subsequent periods of protein feeding is not by any means a demonstration that the *total* endogenous catabolism also remains constant.

There seems to be no method of settling directly or finally the question whether, during a period of protein feeding, the contribution of the tissues to the urinary nitrogen is the same as the total urinary nitrogen excreted on a protein-free ration. It is possible, nevertheless, to throw some light on this question in a more or less indirect way. Both the theory of Sherman and that of Osborne and Mendel assume that the endogenous catabolism would be *diminished* by protein feeding, although the creatinine excretion itself is not thus affected. If either of these theories be correct, it would be expected that the urinary nitrogen excreted on a diet containing a small amount of a protein of high biological value would be considerably less than the urinary nitrogen excreted on a nitrogen-free diet. However, Mitchell [15] has shown that such is not the case, and has cited further evidence in support of Folin's contention of a constant endogenous catabolism. A study of the nitrogen excretion of rats differing widely in weight, but receiving approximately the same amounts of a constant protein mixture, indicated that an

[15] Mitchell, H. H., *J. Biol. Chem.*, 1924, lviii, 873.

approximately constant utilization of the protein mixture, as measured by its biological value, occurred when the endogenous catabolism was assumed to remain constant. Neglecting the endogenous catabolism entirely in this calculation, a procedure that would be justified on the basis of the theory of Sherman or of that of Osborne and Mendel, resulted in very variable estimates of protein utilization. It would seem that the close agreement secured between sets of data seemingly so discordant as regards the utilization of the dietary nitrogen by the assumption of a constant endogenous catabolism, testifies strongly to its essential accuracy. The "constancy" of the endogenous catabolism, as the word is used throughout this discussion, refers more to its independence of the exogenous catabolism of protein rather than to an actual and absolute invariability. Age, size, and possibly other factors associated with "muscular tone" and "vitality" apparently affect the endogenous catabolism as they do the basal heat production.

DOES THE MINIMUM ENDOGENOUS CATABOLISM INVOLVE THE DESTRUCTION OF PROTEIN?

Although the original conclusion of Folin that the endogenous catabolism was distinct from and independent of the exogenous catabolism, appears to be in good agreement with the recorded data of nitrogen metabolism investigations, the conception that the endogenous catabolism results from the breakdown of tissue proteins is difficult if not impossible to reconcile with many known facts. The partial utilization of incomplete proteins, incomplete amino acid mixtures, and even ammonium salts, in covering the maintenance requirement of nitrogen is inconsistent with a belief that the replenishment of endogenous losses involves the synthesis of tissue proteins, which would require a complete aggregate of the indispensable amino acids. McCollum and Steenbock's conception of an incomplete endogenous hydrolysis of tissue proteins, requiring incomplete amino mixtures for the maintenance of the integrity of the tissues, is a makeshift conception, with no foundation in fact or in analogy.

It was suggested by Mitchell, Nevens and Kendall [10] in 1922 that the facts enumerated above could be explained in much less awkward fashion by dispensing entirely with the conception that protein disintegration is an essential phase of cellular activity. Going back to Folin's original work, the striking difference between the end-products of the endogenous catabolism and of the exogenous catabolism may be explained as readily on the basis of a fundamental difference in the *materials* catabolized, as in the *processes* of catabolism, or in the *tissues*

in which the catabolism occurs. Furthermore, there are many indications that the minimum endogenous catabolism, at least, does not involve protein destruction.

Before proceeding with the argument, it is necessary to point out the fact that tissue catabolism, as it relates to nitrogenous compounds, is evidently of two distinct types. One type is the endogenous catabolism of Folin, to which the tissues are reduced by continued subsistence upon nitrogen-free diets containing an adequate amount of energy. In this condition the composition of the urine possesses the characteristics described by Folin. The other type represents an *acceleration* of tissue breakdown brought about, by an inadequate energy intake, by certain diseases, such as exophthalmic goiter and diabetes, or by certain poisons. The end-products of this type of endogenous catabolism resemble those of the exogenous catabolism in consisting largely of urea and in containing no creatinine. This accelerated type of endogenous catabolism becomes prominent during fasting, in which condition the composition of the urine is quite similar to that resulting from a normal protein-containing dietary. In the discussion that follows, the first type will be referred to as the "minimum" endogenous catabolism, and the second type as the "accelerated" endogenous catabolism. The first type is apparently fairly constant in intensity and is largely independent of dietary factors. The second type is a sporadic phenomenon resulting from under-nutrition, disease, or other abnormal factors. It obviously involves the destruction of tissue proteins, since tissue emaciation results. The first type, however, does not appear to involve protein destruction for reasons that will now be considered.

AUTOLYSIS AND THE MINIMUM ENDOGENOUS METABOLISM

Although other methods of protein disintegration in animal tissues have been suggested or demonstrated under laboratory conditions,[16] the only method known to occur *in vivo* is hydrolysis by tissue proteases. This is known as autolysis, and has been investigated actively for a long time. In an excellent summary of the information accumulated on this subject, Bradley,[17] who has himself made many valuable contributions, has the following to say:

"To summarize, we find that the enzyme mechanism of autolysis comprises at least two groups of proteolytic enzymes. One acts only in acid media and converts the acid tissue proteins into primary cleavage products. It is active between pH 7 and 2.6, and its optimum is about

[16] Clifford, W. M., *Biochem. J.*, 1924, xviii, 669. Hopkins, F. G., *ibid.*, 1925, xix, 787.
[17] Bradley, H. C., *Physiol. Revs.*, 1922, ii, 415.

4.5. The second attacks only the primary cleavage products of the proteins, producing the amino acids. It is active between pH 8 and 3, and seems to be identical with the erepsin of the intestinal tract.

"From the data presented above we may form what is probably a fairly accurate hypothesis for relating autolysis to the various normal and pathological atrophies. The normal tissue cell is maintained at the reaction of the blood, or pH 7.4. So long as its metabolic processes remain in equilibrium with its blood and lymph supply there is no accumulation of carbon dioxide, sulphuric acid, phosphoric acid or acids of intermediary metabolism. Neutralization of these acids, further oxidation of some of them, diffusion out of acid ions and diffusion in of basic ions goes on at such rates that the resultant reaction remains a constant.

"Under these conditions the cell proteins are in the form of base-protein salts. The primary protease is inactive, the ereptase active but inoperative, and the amino acids present in the cell are in equilibrium with the proteins and in diffusion equilibrium with the amino acids of the blood. Any prolonged increase of metabolism within the cell without compensatory increase of the circulation about it would lead to an acidotic shift within the cell. Such shift, resulting from excessive acid production, leads to a rise in the H-ion and a change from base- to acid-protein. Primary cleavage results and the products are further disintegrated by the ereptase. The excess amino acids diffuse out into the blood and lymph until an equilibrium is again attained. The net result of these steps is a cell of decreased mass and presumably of decreased metabolism, and the decrease continues until accurate adjustment between the cell and its blood supply is reëstablished. Any increase in acid production in cell or tissue beyond the capacity of the buffer mechanism to immediately dispose of must automatically tend to atrophic changes of mass."

According to Bradley, "The native tissue proteins appear undigested at pH 7 after months, and the amino acid production is negligible." It is evident, therefore, that autolysis of tissue proteins is a sporadic and abnormal phenomenon, bearing no relation to the minimum endogenous catabolism. The presumption is, therefore, that the latter does not involve protein disintegration.

THE SIGNIFICANCE OF THE INDEPENDENCE IN THE EXCRETION OF THE END-PRODUCTS OF THE MINIMUM ENDOGENOUS METABOLISM

If the minimum endogenous catabolism represents protein disintegration, as is generally assumed, one would expect the rates of elimination of the various nitrogenous end-products of this catabolism to be

interdependent in their variations. However, this does not seem to be the case. The creatinine elimination is remarkably uniform from day to day and from hour to hour, and does not seem to be appreciably affected by any normal condition of nutrition. In marked contrast with this is the wide and unaccountable fluctuation to which the uric acid excretion is subjected, not only from day to day, but from hour to hour. As Folin has said of the endogenous uric acid in the urine: "The metabolic processes that determine the uric acid excretion may therefore be said to be in relatively unstable equilibrium." Also, many investigators have shown that food free from purine precursors, particularly protein food, may markedly raise the level of endogenous uric acid excretion. Thus, Lewis, Dunn and Doisy [18] have shown that proteins and amino acids may increase the hourly elimination of uric acid 100 or 200 per cent with no perceptible effect on the excretion of creatinine. The independence of the excretion of these two metabolites in pathological states has also been clearly shown.

In their study of the endogenous metabolism of pigs, McCollum and Hoagland [19] have demonstrated that the minimum nitrogenous catabolism, to which the pig is reduced on a diet of starch and a salt mixture of a potentially alkaline character, may be accelerated in different ways in response to different stimuli. When a mineral acid is added to the basal diet the endogenous catabolism is accelerated entirely by an increase in ammonia excretion, the excretion of urea and creatinine being unaffected. When benzoic acid is added to the basal ration in small to moderate amounts, the total endogenous catabolism may not be accelerated at all. However, a portion of the nitrogen which, in the absence of benzoic acid, would appear as urea, is diverted to the synthesis of hippuric acid, no other appreciable effect on metabolism resulting. The urea nitrogen cannot apparently be decreased to a lower level than about 20 per cent of the total, a synthesis of glycine from other sources occurring with increasing doses of benzoic acid. With the ingestion of excessive amounts of benzoic acid, there is a marked acceleration in the total nitrogenous catabolism with an increased excretion of ammonia. Furthermore, Lewis and Karr [20] have shown that the ingestion of sodium benzoate affects the hourly excretion of uric acid, causing a marked diminution (50 to 70%) in the first 4 hours after benzoate administration, while no effect on creatinine was noted.

The evidence cited to the effect that the rate of elimination of those end-products of the endogenous catabolism that are not obviously inter-

[18] Lewis, H. B., Dunn, M. S., and Doisy, E. A., *J. Biol. Chem.*, 1918, xxxvi, 9.
[19] McCollum, E. V., and Hoagland, D. R., *J. Biol. Chem.*, 1913-14, xvi, 299, 317, 321.
[20] Lewis, H. B., and Karr, W. G., *J. Biol. Chem.*, 1916, xxv, 13.

convertible in the body, may be varied independently of each other, may be interpreted as meaning that the precursors of these metabolites are to be found among the non-protein compounds of tissues, each of which probably has some specific function to perform and is catabolized at varying rates in response to variations in the function it serves. The evidence is not readily explained by the ordinary view that the precursors of the endogenous metabolites are united firmly in a protein molecule.

THE AVAILABILITY OF ENDOGENOUS CYSTINE FOR DETOXICATION OF FOREIGN BODIES

Thomas and Straczewski [21] have secured experimental evidence indicating that the minimum endogenous catabolism does not involve the catabolism of cystine, a constant constituent of protoplasmic proteins. If a dog on a meat diet is given brombenzene *per os,* it is excreted in the urine in part as *p*-bromphenyl mercapturic acid, a conjugation product of brombenzene and cysteine.[22] Under such conditions cysteine, a reduction product of cystine, has been used by the body in the detoxication of a foreign compound. However, Thomas and Straczewski found that if the dog is reduced to its minimum endogenous catabolism by continued feeding of a protein-free diet, the administration *per os* of brombenzene does not lead to a production of *p*-bromphenyl mercapturic acid. This interesting experimental finding has been confirmed by Muldoon, Shiple, and Sherwin.[23] Even the subcutaneous administration of brombenzene, to insure contact of the foreign body with the tissues in which the endogenous catabolism is occurring, did not induce a conjugation with cysteine. To prove that an animal in a condition of specific nitrogen hunger still possesses the ability to perform this conjugation, Kapfhammer [24] gave such a dog brombenzene by mouth and cystine subcutaneously. In this case *p*-bromphenyl mercapturic acid was produced.

From this evidence Thomas concludes that cystine is not liberated from the tissue protein in its breakdown in the course of the minimum endogenous catabolism. However, since the possibility of the body's conserving some amino acids in the course of tissue autolysis has not been established or even indicated directly by experimental investigation, the evidence cited would seem to be more satisfactorily explained on the assumption that the minimum endogenous metabolism does not involve protein disintegration at all, but is related entirely to the metabolism of the non-protein nitrogenous constituents of the tissues. Possibly gluta-

[21] Thomas, K., and Straczewski, H., *Arch. Anat. Physiol., Physiol. Abt.,* 1919, 249.
[22] Jaffé, M., *Ber. chem. Ges.,* 1879, xii, 1092. *Also,* Baumann, E., and Preusse, C., *ibid.,* 1879, xii, 806.
[23] Muldoon, J. A., Shiple, G. J., and Sherwin, C. P., *J. Biol. Chem.,* 1924, lix, 675.
[24] Kapfhammer, J., *Z. physiol. Chem.,* 1921, cxvi, 302.

thione, the cysteine-containing peptide of the tissues, is not catabolized in such a way as to liberate cysteine for purposes of detoxication.

THE SIGNIFICANCE OF THE ENDOGENOUS CREATINE AND CREATININE OF THE URINE

The facts concerning the excretion of creatinine and creatine in the urine also appear to be consistent with the theory that the minimum endogenous catabolism relates to the non-protein nitrogenous constituents of the tissues rather than to the proteins of the tissues. A review of the physiology of creatine and creatinine has recently been prepared by Hunter.[25] The following review will, therefore, be restricted to those investigations more directly bearing upon the particular question under discussion.

The excretion of creatinine is remarkably constant for the same individual from day to day as well as from hour to hour. This is true whether the individual is fasting for short periods, or is subsisting on a nitrogen-free diet, or on a diet containing even large amounts of protein provided only that it be free of creatinine and creatine. The creatinine excretion is therefore independent of the amount of total nitrogen appearing in the urine. It is also independent of the urine volume. Thus Marshall [26] has found that the diuresis induced by the ingestion of large quantities of water, definitely increased the urea excretion, but had no measurable effect on the excretion of creatinine. With increases of twenty-fold in urine volume, the urea excretion could be increased two-fold, while the creatinine excretion was constant.

The creatinine excretion of pathological subjects is usually low in proportion to body weight, especially if extreme muscular weakness is involved. This is particularly true in exophthalmic goiter,[27] myasthenia gravis,[28] myotonia congenita, muscular dystrophy,[29] and diabetes mellitus.[30] The creatinine excretion is definitely increased in fevers, induced artificially or by infection.[31]

The general conclusion that the creatinine constantly excreted in the urine is derived from the creatine of muscle and other tissues may be said to amount to a demonstration at the present time. This demonstration cannot be said to rest upon any particular investigation, or any

[25] Hunter, A., *Physiol. Revs.*, 1922, ii 586. *See also* Hammett, F. S., *J. Biol. Chem.*, 1924, ix, 347; Chanutin, A., *ibid.*, 1926, lxvii, 29; Edgar, G., and Shiver, H. E., *J. Amer. Chem. Soc.*, 1925, xlvii, 1179.

[26] Marshall, E. K., Jr., *J. Pharm. Exper. Therap.*, 1920, xvi, 141.

[27] Shaffer, P. A., *Amer. J. Physiol.*, 1908, xxiii, 1.

[28] Pemberton, R., *Amer. J. Med. Sci.*, 1910, cxxxix, 816.

[29] Spriggs, E. L., *Biochem. J.*, 1907, ii, 206; Levene P. A., and Kristeller, L., *Amer. J. Physiol.*, 1909, xxiv, 45; Janney, N. W., Goodhart, S. P., and Isaacson, V. I., *Arch. Intern. Med.*, 1918, xxxi, 188; Ziegler, M. R., and Pearce, N. O., *J. Biol. Chem.*, 1920, xlii, 581.

[30] Taylor, M., *Biochem. J.*, 1910-11, v, 362; Lampert, D., *Z. klin. Med.*, 1914, lxxx, 498.

[31] Myers, V. C., and Volovic, G. O., *J. Biol. Chem.*, 1913, xiv, 489; Shaffer, P. A., *sec*[27]; McClure, C. W., *Arch. Intern. Med.*, 1918, xxii, 719.

particular line of investigation, but to rest upon the entire mass of work done in the last twenty years, a critical discussion of which will be found in Hunter's review.[25] The difficulty in demonstrating the connection between tissue creatine and urinary creatinine lies in the remarkable constancy in the capacity of the body to dehydrate creatine. This dehydration reaction does not seem to be appreciably amenable to the law of mass action, since neither the ingestion of large amounts of creatine, nor the production of this substance in excess by the disintegration of body tissue, as in inanition and other conditions similarly characterized, affects the output of creatinine to a marked degree. In view of the high resistance of ingested creatinine to catabolism [32] it appears justifiable to interpret differences in excretion as due to differences in production. This interpretation is further justified by the fact that creatinine is one of the most readily eliminated of the nitrogenous metabolites.[33]

The facts in regard to the excretion of creatinine are generally interpreted as indicative of a constant type of metabolism occurring in the tissues, unaffected by diet or by most of the environmental conditions to which an animal is normally subjected. This is the minimum endogenous catabolism of Folin, to which the nitrogenous metabolism of an animal is reduced by continued subsistence on a nitrogen-free diet of sufficient energy value. The conversion of tissue creatine to creatinine appears to be involved solely in this minimum endogenous catabolism and appears to be unaffected by any condition which does not at the same time affect the endogenous catabolism. According to Shaffer: "Creatinin is derived from, and its amount . . . is an index of some special process of normal metabolism taking place largely if not wholly in the muscles. And upon the intensity of this process appears to depend the muscular efficiency of the individual." The reference of creatinine excretion largely to muscle metabolism rather than to general tissue metabolism, seems amply justified by the fact that all but 2 per cent of the body creatine is contained in the muscles.

The excretion of creatine in the urine of adults and adolescents, under normal and pathological conditions, has been the subject of innumerable investigations, in spite of which no conclusion as to its origin or significance in metabolism has received general acceptance. The problem is to some extent clouded by uncertainty as regards analytical methods. Especially in pathological urines, the question may be raised of the correctness of interpreting an increased reducing action of urine after hydrolysis on picric acid in alkaline solution as due to the presence of

[32] Lyman, J. F., and Trimby, J. C., *J. Biol. Chem.*, 1917, xxix, 1; Rose, W. C., and Dimmit, F. W., *ibid.*, 1916, xxvi, 345.

[33] Myers, V. C., and Fine, M. S., *J. Biol. Chem.*, 1919, xxxvii, 239.

creatine, since it is known that acetoacetic acid when present in urines in considerable amounts, lowers the estimation of preformed creatinine and has no effect on the estimation of total creatinine, since it is volatilized during the process of hydrolysis. The net effect of its presence, therefore, would be to indicate a lowered creatinine excretion and a fictitious presence of creatine, or if creatine is actually present, an exaggerated excretion.[34] In view of the demonstrated independence of the excretion of urinary creatine and creatinine, it may be fairly assumed that this error has entered into those investigations, largely of prolonged fasts, in which a close inverse relation has been shown to exist between the excretion of these two substances.

One of the most remarkable facts brought out by investigations on creatinuria is the total independence in the excretion of creatine and its anhydride. The excretion of creatinine may remain constant in the presence or absence of creatine, as in starvation; it may increase markedly with no occurrence of creatine, as in fever,[31] or it may decrease to a value much below normal in the presence of considerable amounts of creatine, as in exophthalmic goiter or diabetes mellitus. McClure [31] has reported urinary analyses indicating that in acute febrile conditions creatinuria or an increased output of creatinine may occur singly or in combination. Janney and Isaacson [35] produced marked disturbance in the nitrogenous metabolism of a thyroidectomized dog by the administration of Kendall's thyroid hormone. The total urinary N practically doubled in amount and continued high even after the hormone administration had ceased. The amounts of urea, ammonia, and purines increased correspondingly. Creatine, absent previous to the injection, appeared in the urine in considerable amounts. However, the creatinine remained absolutely irresponsive to this tremendous metabolic stimulus.

The facts concerning the elimination of creatine are consistent with the theory that it is produced in the tissues from disintegrating protein, whenever the minimum endogenous catabolism is accelerated by any means whatever, such as a disturbance of the acid-base equilibrium of the tissues, or a disturbance of the ability of the tissues to utilize carbohydrate, or a disturbance of the normal activity of the endocrine glands, or the presence of bacterial toxins. On the other hand, the evidence seems to indicate that urinary creatine may have an exogenous source in infants and growing children and in women when fed excessive amounts of certain proteins. The exogenous origin of urinary creatine is considered under the discussion of the metabolism of arginine.

[34] Graham, G., and Poulton, E. R., *Proc. Roy. Soc. London*, 1913, B, lxxxvii, 205.
[35] Janney, N. W., and Isaacson, V. I., *Arch. Intern. Med.*, 1918, xxii, 174.

Considering only the endogenous origin of creatine, it is normally present in the urines of fasting animals, and in general occurs in the greatest amount in the urine of those animals in the poorest nutritive condition at the beginning of the fast; in such individuals the destruction of tissue proteins is more intense than in others possessing a greater store of fat. The apparently erratic occurrence of creatine in the urine of fasting pigs, reported by Steenbock and Gross,[36] is explained on this basis. Those pigs excreting during fasting amounts of nitrogen in the urine very little above the minimum endogenous metabolism, exhibited no creatinuria, while those pigs excreting amounts of total nitrogen in considerable excess of this level, always exhibited creatinuria, in some relation to the total urinary nitrogen. The following data on the five fasting pigs investigated by Steenbock and Gross, and earlier by McCollum and Steenbock,[37] illustrate the point made.

TABLE 42. *Creatinuria in Fasting Pigs.*

From published data of McCollum, Steenbock and Gross.

Weight of Pig	Days of Fast (incl.)	Total Nitrogen in Urine	Per Cent Creatinine Nitrogen in Urine	Creatine in Urine
lbs.		grams		grams
85	7-15	3.11	9.17	0.000
51	4-6	5.47	2.67	0.393
55	4-5	10.50	1.12	0.836
59	3-5	9.25	1.94	0.412
77	6-9	2.97	10.11	0.000

Evidently the first and last pigs, exhibiting no creatinuria were excreting but little more nitrogen in the urine than if they had been on a starch diet, probably due to a high fat condition.

The excretion of creatine during fasting runs roughly parallel with the excretion of total nitrogen. This is particularly well shown in the data of the 31 day fast reported by Benedict,[38] in which the daily excretion of urinary nitrogen was initially low, increased on the third day (probably due to a depletion of glycogen stores), maintained this high level for about 2 weeks, and then sank to a low level at the beginning of the third week. The rate of creatine elimination exhibited much the same variation. The urinary data from the 117 day fast of a dog, reported by Howe, Mattill, and Hawk,[39] show the same relation.

The accelerated endogenous catabolism of fasting may be entirely suppressed by carbohydrate feeding, and it has been repeatedly shown

[36] Steenbock, H., and Gross, E. G., *J. Biol. Chem.*, 1918, xxxvi, 265.
[37] McCollum, E. V., and Steenbock, H., *J. Biol. Chem.*, 1912, xiii, 209.
[38] Benedict, F. G., *Carnegie Inst. Washington, Publ.* No. 203, 1915.
[39] Howe, P. E., Mattill, H. A., and Hawk, P. B., *J. Biol. Chem.*, 1912, xi, 103.

that the administration of carbohydrate to fasting animals leads to the prompt disappearance of creatine in the urine.[40] It may be greatly inhibited by the administration of alkaline salts, and by this means also may the creatinuria be suppressed or greatly diminished.[41, 36] It is not diminished by the ingestion of fats, nor is the elimination of creatine.[40] Experimental results on the effect of protein feeding on the creatinuria induced by fasting are conflicting. Cathcart in his work on fasting men, and Mendel and Rose, in observations on fasting rabbits, observed no marked reduction in the creatinuria as the result of feeding protein. On the other hand, the feeding of protein to fasting dogs promptly leads to the inhibition of creatinuria.[42] In a more recent investigation Rose, Dimmitt, and Cheatham [43] were able to cause the entire disappearance of the creatine in the urine of a fasting man by excessive protein feeding. There is no method of determining the effect of protein feeding on the accelerated endogenous catabolism consequent upon fasting. The attainment of nitrogen equilibrium or even of considerable positive balances does not indicate a suppression of the accelerated endogenous catabolism. The fact that nitrogen equilibrium can be attained at a much lower level of protein intake when carbohydrate is fed than when it is absent from the diet, would indicate the relative ineffectiveness of protein as compared with carbohydrate in sparing tissue protein. Excessive protein feeding, however, would conceivably reduce the endogenous catabolism ultimately to its minimum level. Therefore, the facts concerning the effect of protein feeding on the elimination of creatine induced by inanition are not inconsistent with the theory that the creatine eliminated is an end-product of an endogenous *protein* metabolism.

The occurrence of creatine in pathological urines is in perfect agreement with the theory that whenever the endogenous catabolism is accelerated, whether by acidosis, by carbohydrate deficiency, by degeneration of tissue or its normal involution (as in muscular dystrophy and postpartum resolution of the uterus, respectively), by a disturbance of endocrine function (as in hyperthyroidism and hyperadrenalism), by bacterial toxins (as in numerous acute febrile conditions), or by any other condition, creatine inevitably appears in the urine. In all these instances creatinuria is accompanied by a marked increase in urinary nitrogen, and its intensity varies fairly closely with the excess elimination of total nitrogen. Reference may be made to the work of Cathcart and

[40] Cathcart, E. P., *J. Physiol.*, 1909, xxxix, 311; Mendel, L. B., and Rose, W. C., *J. Biol. Chem.*, 1911-12, x, 213; Wolf, C. G. L., and Osterberg, E., *Biochem. Z.*, 1911, xxxv, 329.
[41] Underhill, F. P., *J. Biol. Chem.*, 1916, xxvii. 141.
[42] Wolf and Osterberg [40]; Benedict, S. R., and Osterberg, O., *J. Biol. Chem.*, 1914, xviii, 195.
[43] Rose, W. C., Dimmitt, F. W., and Cheatham, P. M., *J. Biol. Chem.*, 1916, **xxvi**, 339.

Orr [44] on the effect of the subcutaneous administration of sodium selenite to dogs. A marked increase in total urinary nitrogen was obtained in each experiment, together with a marked increase in creatine excretion. If the greatest increase in urinary nitrogen occurred on the day of injection, the creatinuria was most pronounced on this day. If, as generally occurred, the maximum effect of the injection on the nitrogenous catabolism was delayed for a day or two, the maximum creatinuria was generally similarly delayed. Similarly with the injection of hydrazine in fasting dogs,[45] simultaneous with the appearance of creatinuria there is a marked acceleration of endogenous metabolism indicated by an increase in the total urinary nitrogen.

The opinion somewhat generally held in this country and England, and associated especially with the names of Cathcart, Mendel, and Rose, that the excretion of creatine in the urine is incidental to a disturbance in carbohydrate utilization, does not explain all the facts as well as the theory ascribing creatinuria to an accelerated endogenous catabolism, and assuming, therefore, that creatine is a normal product of the catabolism of the protein of the tissues. Whenever carbohydrate utilization is lowered, or whenever there is a deficiency of carbohydrate in the diet, the endogenous catabolism is accelerated and creatinuria supervenes. In these cases either theory fits the facts. But there are other instances of creatinuria in all of which an accelerated endogenous metabolism occurs, but in which no obvious derangement of carbohydrate utilization exists. The prompt appearance of creatine in the urine on the first day of starvation in human subjects, not infrequently reported in the literature, is a case in point, as well as the decrease in the creatinuria of fasting induced by the administration of alkalies. The marked rise in the creatine excretion of fasting animals just prior to death, which cannot be suppressed by excessive carbohydrate feeding is another illustration. The absence of creatinuria in pigs during inanition at low levels of nitrogen metabolism, also, does not square with the carbohydrate deficiency theory, nor does its occurrence on a diet of starch, fat, and neutral salts [46] on which, however, the endogenous catabolism was accelerated slightly. In the investigations of Steenbock and Gross on pigs, it very frequently happened that the creatinuria induced by fasting was not entirely suppressed, even by several days' feeding of 300 to 500 grams of starch. Only when the endogenous catabolism of nitrogen was reduced to, or very nearly to, the minimum did the creatinuria

[44] Cathcart, E. P., and Orr, J. B., *J. Physiol.*, 1914, xlviii, 113.
[45] Underhill, P. P., and Baumann, E. J., *J. Biol. Chem.*, 1916, xxvii, 151; MacAdam, W., *Biochem. J.*, 1915, ix, 229.
[46] McCollum, E. V., and Hoagland, D. R., *J. Biol. Chem.*, 1913, xvi, 305.

disappear. This fact seems inconsistent with the carbohydrate deficiency theory, but appears to be explainable by the theory that creatine is a normal product of an accelerated endogenous catabolism. The frequent occurrence of creatine in the urine in acute febrile conditions may also be cited in this connection.

Experimental evidence that has been interpreted against the theory that creatinuria is a result of tissue destruction has been obtained by Benedict and Osterberg.[42] These investigators were concerned primarily with testing the validity of the theory, first put forth by F. G. Benedict and later elaborated by Myers and Fine [47] to the effect that the creatinuria of fasting is due to the disintegration of muscle tissue and the liberation of its creatine. Working on phlorhizinized dogs, Benedict and Osterberg were unable to affect markedly the elimination of creatine by the feeding of protein, though there was considerable retention of the food nitrogen as indicated by nitrogen balances; in one experiment, a positive balance resulted. An analysis of the muscle of two of the dogs showed a creatine content slightly above the average figure reported by Myers and Fine for dog muscle.[48] These results are interpreted as showing "that in the dog a high creatine elimination in the urine may be maintained which is wholly independent of body tissue destroyed" and "that the creatine eliminated did not have its origin in preformed creatine of the muscular tissue." This conclusion appears to be based on the assumption that the reduction in the nitrogen lost from the body, or even the attainment of nitrogen equilibrium, means that the accelerated tissue catabolism induced by phlorhizin has been reduced or even suppressed entirely. However, this assumption is not justified. The reduction of a negative nitrogen balance, or its conversion into a positive nitrogen balance, means simply that whatever tissue losses the body is sustaining are being partially or completely *compensated for* by the food nitrogen consumed. To prove that the endogenous metabolism of the dogs had been reduced to the minimum and the phlorhizin effect eliminated by protein feeding, it would be necessary to show that nitrogen equilibrium could be established at as low a level of intake with phlorhizinized dogs as with dogs not so treated. A nitrogen balance simply indicates the *net* gain or loss of nitrogen to the body, and can throw no light upon the question whether the endogenous catabolism has been suppressed, or whether it has been unaffected, the losses thus incurred having been partially or wholly replaced from the intestinal tract.

In further support of their contention that the body tissue of phlor-

[47] Myers, V. C., and Fine, M. S., *J. Biol. Chem.*, 1913, xv, 283.
[48] Myers, V. C., and Fine, M. S., *J. Biol. Chem.*, 1913, xiv, 9.

hizinized dogs may be spared in the true sense of the word by protein feeding, Benedict and Osterberg point to the very slight drop in creatinine elimination during several days' feeding of large amounts of protein. However, if the accelerated endogenous catabolism of phlorhizin diabetes were entirely unaffected by protein feeding, and if the losses were largely replaced, the same would be true. Rose [49] has obtained much the same results with depancreatized dogs as Benedict and Osterberg obtained with phlorhizinized dogs, though in the experiments of Rose the creatine elimination could be perceptibly reduced by carbohydrate feeding. The fact that protein feeding in the fasting dog can cause the disappearance of creatine from the urine, while with phlorhizinized and depancreatized dogs no such result can be secured, constitutes, according to Rose, "conclusive proof of the dependence of the creatine elimination upon the carbohydrate utilization." Such facts, however, are not inconsistent with the theory that creatine appears in the urine as the result primarily of an accelerated endogenous catabolism, since it is quite conceivable that dietary protein can spare body protein in fasting but not in experimental diabetes. The accelerated tissue catabolism of fasting is due solely to a lack of adequate nourishment, while in diabetes it is due to causes unrelated to diet.

It would seem that the question whether the creatinuria of experimental diabetes is due primarily to an accelerated endogenous catabolism or to a disturbance in carbohydrate utilization might be settled by the feeding of sugar to completely phlorhizinized or depancreatized animals. Ringer [4] has shown that the administration of glucose to a completely phlorhizinized dog, reduced very greatly the nitrogen catabolism, while with depancreatized animals no such effect was secured. Therefore, if the administration of sugar has no effect on the creatinuria in either type of diabetes, the conclusion would be justified that the primary cause of the creatinuria was defective carbohydrate metabolism. On the other hand, if the creatinuria of phlorhizin diabetes could be suppressed by the feeding of glucose, while in pancreatic diabetes it was unaffected by sugar feeding, it would be justifiable to conclude that the primary cause of creatinuria is an accelerated endogenous catabolism. Apparently no such crucial experiment has actually been performed. The nearest approach to it is an experiment by Cathcart and Taylor [50] on the effect of phlorhizin injection on the nitrogen metabolism of a dog on a mixed diet containing variable amounts of carbohydrate. These investigators showed that, if the diet of a dog contains sufficient carbohydrate to

[49] Rose, W. C., *J. Biol. Chem.*, 1916, xxvi, 331.
[50] Cathcart, E. P., and Taylor, M. R., *J. Physiol.*, 1910-11, xli, 276.

maintain its body weight, the injection of phlorhizin does not induce an increased excretion of nitrogen in the urine nor does creatine appear in the urine, though a marked glycosuria does occur. When smaller amounts of carbohydrate were fed, creatinuria supervened simultaneously with an increased nitrogenous catabolism.

The theory of the endogenous origin of urinary creatine founded mainly on the work of Myers and Fine [47] is that creatine eliminated in the urine originates from the preformed creatine in the muscles, which is liberated by the disintegration of muscle tissue. In a comprehensive investigation on the creatinuria of starving rabbits and on the changes in the creatine content of the muscle tissue and the entire carcass induced by fasting, they conclude that the creatine eliminated in the urine very largely accounts for the creatine lost from the tissues, and that the creatine content of the muscle is clearly dependent upon the amount and rate of creatine excretion in the urine. They conclude that, in the light of these data, "there can be little doubt as to the origin of urinary creatine in pathological conditions associated with malnutrition and loss in weight, especially in view of the observations of Chisolm [51] that there is a decrease in the content of muscle creatine, at least in some of these conditions."

Such computations as are attempted in this paper, correlating changes in the creatine content of the body or the muscle with the excretion of creatine in the urine, are based upon the tacit assumption that creatine formation and destruction in the body are either negligible or very evenly balanced. However, with 15 out of 17 rabbits the estimated loss of creatine from the body was considerably exceeded by the total creatine (including creatinine) eliminated in the urine, indicating a formation of creatine; in some cases the total creatine excreted was practically double the estimated loss of creatine from the body. Furthermore, in prolonged starvation a marked loss in the creatine content of muscle was observed, 30 to 35 per cent in several cases. If the urinary creatine were derived from the preformed muscle creatine, the decrease noted in the concentration of the latter would be the result of a steadily increasing percentage of creatine plus creatinine nitrogen in the urine as the length of the fasting period increases. However in the entire series of results the percentage of the total urinary nitrogen appearing as creatine, plus creatinine was remarkably constant, whether the animal was killed after a fast of one week or of three weeks.

This constant relation observed between the total urinary nitrogen and the creatine plus creatinine nitrogen for rabbits fasting from 6 to

[51] Chisolm, R. A., *Biochem. J.*, 1912, vi, 243.

27 days, may be interpreted as evidence in favor of the conclusion that urinary creatine is excreted in proportion to, and as an end product of, an accelerated endogenous catabolism. In fasting the total endogenous catabolism would be measured by the total urinary nitrogen, and would be proportional to the excretion of creatine, representing the accelerated (protein) catabolism of fasting, and of creatinine, representing the minimum (non-protein) endogenous catabolism.

There is no evidence to indicate the fact of the preformed creatine in muscle undergoing the accelerated catabolism induced by fasting. It is quite possibly catabolized, since, in rabbits fed almost exclusively on carbohydrate, as marked a decrease in the muscle content of creatine occurs as in fasting,[52] though the creatine elimination is much less. The estimated loss of body creatine in these experiments of Myers and Fine was much larger than the total creatine excretion.

It is true that the creatinuria of children and the intermittent creatinuria of women are not readily explainable on the basis of the theory advocated here that the endogenous excretion of creatine in the urine is an indication of the destruction of tissue protein, as contrasted with the catabolism of the non-protein nitrogenous tissue constituents characteristic of the minimum endogenous metabolism. However, so little is known of the causes of these types of creatinuria that attempts to reconcile them with any theory have not been appreciably successful.[25]

THE SIGNIFICANCE OF THE NON-PROTEIN NITROGENOUS CONSTITUENTS OF THE TISSUES

The constancy of the minimum endogenous catabolism and its probable relation to the non-protein constituents of the tissues, raises the question of the significance of these constituents and of the factors concerned in their catabolism. In this connection the experimental findings of Mitchell, Nevens, and Kendall [10] on the effect of non-protein feeding on the concentration of these constituents in the tissues would seem to be of considerable import. It was found that the concentration of non-protein nitrogen and sulfur and of amino nitrogen was approximately the same in the tissues of rats reduced to the minimum endogenous catabolism by continued feeding on a nitrogen-free ration as in the tissues of rats on normal rations in the post-absorptive period.

This approximate constancy of the concentration of the total non-protein nitrogen and sulfur and of amino nitrogen of the tissues, regardless of the type or the intensity of the catabolic processes resulting in nitrogenous or sulfur-containing end-products, would seem to be a

[52] Myers, V. C., and Fine, M. S., *J. Biol. Chem.*, 1913, xv, 305.

matter of significance to any theory of the endogenous catabolism. In explaining a similar situation as related to the amino-acid concentration of the tissues as influenced by fasting, Van Slyke and Meyer [53] put forward two possibilities as to the origin and function of the free amino acids of the tissues: first, that the amino-acids might serve as a reserve energy supply or as a reserve of tissue-building material; and, second, that their presence in the tissues might be dependent simply upon the fact that they are intermediate steps in the construction and the breakdown of tissue proteins, originating either from absorbed food products or autolyzing tissue protein. From the fact that starvation does not decrease the concentration of amino acids in the tissues, these investigators conclude that the second possibility is correct.

However, since the concentration of amino acids in the tissues is not diminished on continued feeding with a non-protein ration, which may be presumed to reduce the protein catabolism to a minimum while affording no building materials for an anabolism of protein, it does not seem probable that the presence of amino acids in the tissues is merely incidental to protein metabolism. Furthermore it seems to be true that intermediary products of metabolism do not normally accumulate to any considerable extent in the tissues, as witness the absence of demonstrable amounts of the hydroxy and keto acids that must be produced in the breakdown of glucose, fatty acids, and amino acids; nor do even the end-products of metabolism accumulate normally to any great extent.

It is fair to presume, therefore, that the free amino acids of the tissues are not functioning as reserve material, nor are they present merely as intermediary steps in the synthesis and disintegration of protein, but that they are performing some distinct and important function in the life of the tissues, since such an effective mechanism exists for maintaining their concentration constant. Their remarkable constancy in the muscle fluids of migrating salmon is also significant in this connection.[54] Their possible function in the maintenance of osmotic pressure within the cell [55] and particularly within the nucleus, which seems to be free from inorganic electrolytes, may also be cited.

The data obtained in the investigation of Mitchell, Nevens and Kendall also indicate that the total non-protein nitrogen and sulfur are not definitely affected by the type or intensity of the protein metabolism. Probably most of the substances contributing to the non-protein fraction of the tissue nitrogen and sulfur are performing specific functions in metabolism.

[53] Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 1913-14, xvi, 231.
[54] Greene, C. H., *J. Biol. Chem.*, 1919, xxxix, 457.
[55] Collip, J. B., *J. Biol. Chem.*, 1920, xlii, 227.

The universal presence of creatine in vertebrate muscle, the fact that it is not an obligate stage in the catabolism of arginine or any other known amino acid, its differential occurrence in different tissues, and its extremely small concentration in the blood as compared to its concentration in the tissues, constitutes strong presumptive evidence that it is serving a function in tissue metabolism. In this connection, Hunter [25] has summarized the significant facts concerning the occurrence of creatine in animal tissues in the following words:

"The creatine content of muscle is not the same at all ages, but rises steadily during fetal and postnatal growth till it attains the maximum characteristic of the adult. There is more creatine in the quickly contracting pale muscle than in the slowly contracting red, a difference exhibited alike by mammal and by bird. The muscle creatine of warm-blooded animals is, generally speaking, higher than that of the more sluggish cold-blooded, although some of the data for fish supply striking exceptions. Smooth muscle has a very much lower concentration of creatine than striped; and the gravid uterus a higher one than the non-gravid. All these facts point in one direction. They indicate very strongly that creatine is a substance with a useful function, a function in some way connected with that capacity for rapid and powerful contraction which is the most important property of striated vertebrate muscle." The established relation between muscle tension or "tonus" and the concentration of creatine [56] is another fact bearing the same significance.*

Similarly with carnosine. This substance is a dipeptide containing an amino acid, β-alanine, not thus far found in proteins, and is extremely resistant to tissue proteases,[57] both facts arguing for a purposive synthesis in muscle cells. In the same category may be placed the autoxidizable dipeptide discovered by Hopkins,[58] consisting of cysteine and glutamic acid. Apparently this compound performs important functions in the chemical dynamics of the cell. The constancy of the concentration of non-protein sulfur in the tissues, regardless of the intensity of catabolism, is consistent with this view.

The relation of the endogenous catabolism to these compounds—and there are probably many others that may be so classed—is an interesting

[56] Riesser, O., *Z. physiol. Chem.*, 1922, cxx, 189; Looney, J. M., *Amer. J. Physiol.*, 1924, lxix, 638; Constabel, Fr., *Biochem. Z.*, 1921, cxxii, 152.

* Other evidence has lately been adduced in support of the belief that creatine performs an important functional rôle in muscle. Tiegs (*Australian J. Exp. Biol. Med. Sci.*, 1925, ii, 1) has found that creatine exists in muscle in a diffusible and in a non-diffusible form, that the latter increases at the expense of the former during prolonged contraction, and that it is formed at a rate proportional to the formation of lactic acid. Fiske and Subbarow (*Science*, 1927, lxv, 403) have secured evidence of a "phosphocreatine" in muscle, a labile phosphorus compound of creatine apparently identical with the non-diffusible creatine substance of Tiegs. The dissociation of this compound into inorganic phosphate and free creatine is brought about by stimulation of the muscle, while its resynthesis occurs during the recovery phase.

[57] Baumann, L., and Ingvaldsen, T., *J. Biol. Chem.*, 1918, xxxv, 263.

[58] Hopkins, F. G., *Biochem. J.*, 1921, xv, 286.

subject of speculation concerning which little definite information is available. Being soluble and diffusible, and, as a class, less stable to oxidation than the cell proteins themselves, one might suppose that their loss from the tissues by purely physical means, or their destruction by catabolic agents, would be more constant, certainly more inevitable, than the loss of the proteins in the cell. However, their destruction does not seem to be closely related to the oxidative phases of catabolism, since extreme variations in tissue oxidations may occur with no effect on the minimum endogenous catabolism of nitrogenous constituents.

If the non-protein nitrogenous substances are continually being lost to the tissues as an incidental consequence of metabolism, it is conceivable that the losses may be replaced from a variety of sources, even from such simple substances as ammonium salts. Possibly any one of the amino acids may be used in the synthesis of one or more of these compounds, either as an obligate or a facultative precursor. Some of them may require some one particular precursor, such as tryptophane, tyrosine, cystine, or histidine, so that such amino acids are indispensable to the maintenance of life. Some of the amino acids that are essential in the synthesis of cell proteins, may not be obligate precursors of any of these nitrogenous extractives, and hence would not be essential for maintenance. Such considerations involve the conception that the requirements of amino acids for the maintenance of the integrity of the tissues are "particulate" not "aggregate" in character, being thus opposed to the requirements for growth. For this reason the losses due to the minimum endogenous catabolism may be *partially* replaced by incomplete proteins, or amino acid mixtures, or even by ammonium salts, sources of nitrogen that would not maintain life no matter how much was consumed. In this respect the requirement of amino acids for maintenance has been compared with the requirement of inorganic ions.[59] On the other hand, growth can never occur unless a certain minimum assortment of amino acids are available.

Under conditions of normal nutrition, one would expect, on the basis of this theory, that these "particulate" requirements would be covered more efficiently by dietary protein than would the "aggregate" requirements of growth, since deficiencies of the dietary protein in one amino acid would not limit the utilization of others. This, Mitchell [60] has shown to be true.

[59] Mitchell, H. H., *J. Biol. Chem.*, 1916, xxvi, 231.
[60] Mitchell, H. H., *J. Biol. Chem.*, 1924, lviii, 905.

HOW IS THE CONCENTRATION OF THE NON-PROTEIN NITROGENOUS CONSTITUENTS OF THE TISSUES MAINTAINED ON NITROGEN-FREE DIETS?

The conditions obtaining in specific protein inanition, *i.e.*, non-protein feeding, are more difficult to reconcile with the theory. When no protein is being consumed, the losses of nitrogen incurred by the constant breakdown of the non-protein nitrogenous constituents of the tissues are evidently replaced from some source, since the concentration of these tissue constituents does not decrease. It may be that they are replaced at the expense of tissue protein by a process other than those of autolysis. Or, if there is a storage of protein in the body, such as is claimed for the liver, some specific method of disintegration that can be put into effect under normal tissue conditions may be available. In such cases, it is necessary to assume that the nitrogen thus degraded can be used without appreciable waste in replacing these essential non-protein constituents, since the urinary nitrogen of an animal on adequate amounts of a nitrogen-free diet represents essentially only the minimum endogenous catabolism.

In this connection, the significance of the functioning of the digestive glands has been emphasized by Kestner.[61] Kestner has estimated, as accurately as available data permit, that the amount of nitrogen-containing organic matter (exclusive of the mucin in the stomach and the bile salts and pigments) secreted per day in the digestive fluids is about 50 grams for man. He concludes that approximately as much nitrogen is secreted into the digestive tract as is required per day for maintenance. Hence the nitrogen requirement of the human body relates for the most part to the reconstruction of the digestive glands.

However, the activity of the digestive glands is variable, depending upon the amount and character of the food consumed [62] (see also p. 234). Hence an apparently constant requirement for nitrogen can hardly be accounted for by reference to such a variable function as this. The point is well illustrated by reference to some data concerning the nitrogen metabolism of rats on a nitrogen-free diet reported by Mitchell [63] and included in Table 43.

Six rats of quite variable weight were fed *ad libitum* on a nitrogen-free ration. After a preliminary period of 3 days, the urine and feces were collected quantitatively for 7 days. At the end of this time the food intake was restricted to an amount equal to their requirement for maintenance of weight as determined previously. A 7-day collection period at this lower level was then undertaken with no transition period.

[61] Kestner, O., *Z. physiol. Chem.*, 1923, cxxx, 208.
[62] Mitchell, H. H., *Bul. Natl. Res. Council*, 1926, xi, Part 1, No. 55, pp. 21-30.
[63] Mitchell, H. H., *J. Biol. Chem.*, 1924, lviii, 880.

TABLE 43. *Influence of the Amount of Food Consumed on the Excretion of Fecal Nitrogen with Rations Practically Nitrogen-Free.*

Rat No.	Average Weight	Daily Food Intake	Daily Excretion of Urinary Nitrogen	Daily Excretion of Fecal Nitrogen	
				Total	Per Gram of Food
	grams	grams	mgms.	mgms.	mgms.
1.....	218	11.32	24.5	24.3	2.15
	213	7.38	28.6	18.3	2.48
2.....	266	14.24	26.6	35.5	2.49
	257	8.67	37.7	21.7	2.51
3.....	162	10.41	13.2	18.9	1.81
	156	6.00	22.2	11.3	1.88
4.....	145	9.61	12.9	21.0	2.19
	139	6.00	17.4	11.3	1.88
5.....	149	8.73	21.0	15.5	1.78
	143	5.93	22.0	10.7	1.78
6.....	69	5.00	8.3	9.5	1.90
	65	3.60	9.3	5.5	1.53

As will be noted on reference to the table, the average daily excretion of fecal nitrogen dropped in all cases in the second period, and, as the last column of figures shows, the decrease was more or less closely proportional to the decrease in food consumed. Evidently the activity of the digestive glands was less in the second period than in the first. The excretion of urinary nitrogen, however, increased in the second period, proving conclusively that the minimum endogenous catabolism of nitrogen bears no close relation to the activity of the digestive glands.

However, it is probable that the fecal nitrogen excreted on a nitrogen-free diet represents only a fraction of the nitrogenous material actually present in the digestive juices as secreted. The remainder has been digested and absorbed and in all probability it is available for the replacement of the non-protein nitrogenous constituents of the tissues, the catabolism of which apparently accounts for the urinary nitrogen excreted under such conditions.

The digestive glands may thus minister to the tissues in specific protein inanition, but the ultimate source of the nitrogenous material of the digestive juices is unknown. If it comes mainly from blood proteins, the problem of the maintenance of the tissues is removed again to the location of the synthesis of these proteins. If it comes from blood amino acids, the activity of the digestive glands has not essentially clarified or simplified the problem under discussion. Further speculation along these lines seems unprofitable.

The replacement of the red cells of the blood is a factor to be considered in this problem. Apparently blood is one tissue that has a "wear-

and-tear quota" in the literal sense of the term. Ashby's work [64] indicates that in human beings erythrocytes are constantly being destroyed at the rate of about 3 per cent per day. The products of their destruction in part find their way into the bile, in small part into the urine, while probably the largest fraction would be available for the replenishment of the digestive glands and the other tissues. However, unless a progressive anemia is an accompaniment of specific protein hunger, the question of the ultimate replacement of endogenous losses as they relate to the blood is removed to the hematopoietic organs. Again further speculation becomes unprofitable.

DETERMINATIONS OF THE MINIMUM ENDOGENOUS CATABOLISM

Many investigations have been reported in the literature in which the minimum endogenous excretion of nitrogen in the urine was determined, either as an object in itself, or as an incidental datum to other objects. In view of the close relation undoubtedly existing between this minimum excretion and the protein requirements, it is of considerable practical interest to study these results.

A number of such results have been reported for human subjects. The values assembled in Table 44 have been selected largely by Thomas, with subsequent additions of Smith and the authors. In the selection of these results, the lowest daily excretions of urinary nitrogen on rations exceedingly low in this element have been chosen. It will be seen that a good deal of variation, from 0.0594 gram down to 0.0241 gram of nitrogen daily per kilogram of body weight, exists among these determinations, due, apparently, not so much to individual idiosyncrasies as to the merely approximate success in most of the experiments in reaching the actual endogenous level of excretion.

The most successful of these investigations are undoubtedly those of Smith and of Deuel, Sandiford, Sandiford and Boothby, the most recent ones reported. The diet used in Smith's experiment was estimated to contain only 0.33 gram of nitrogen, while containing 3157 calories on the 24th day of the experiment. The collection of urine for this day contained 1.58 grams of nitrogen (0.0242 gram per kilogram body weight) of which only 22.1 per cent was in the form of urea; 35.4 per cent was in the form of creatinine, 21.0 per cent was undetermined, 14.5 per cent was present as ammonia, and 7.0 per cent as uric acid.*

[64] Ashby, W., *J. Exper. Med.*, 1919, xxix, 267; *ibid.*, 1921, xxxiv, 127.

* On the basis of his data, Smith has proposed the theory that the urea + ammonia nitrogen in the urine not traceable to the dietary nitrogen, can be entirely accounted for by "the discarded amino groups from arginine molecules which are used for the formation of creatinine." In support of this hypothesis it is shown that if the estimated nitrogen intake be subtracted from the urea + ammonia nitrogen of the urine, on the assumption that it would be so excreted, the remainder is in close agreement with one-third of the creatinine nitrogen. It is assumed that

TABLE 44. *Recorded Determinations of the Minimum Endogenous Urinary Nitrogen of Human Subjects.*

Day of Experiment	Total Urinary N	Body Weight	Nitrogen per Kilo	Investigator
	gms.	kgms.	gms.	
10	3.8	64.0	0.0594	Folin [1]
4	3.76	69.7	0.0539	Langergren [2]
5	3.5	70.5	0.0497	Folin [1]
4	3.04	62.4	0.0487	Landergren [2]
5	2.7	55.7	0.0485	Folin [1]
8	3.12	63.5	0.0480	Klemperer [3]
7	3.34	71.3	0.0468	Landergren [2]
7	2.42	57.5	0.0421	Roehl [4]
8	2.89	71.0	0.0407	Kocher [5]
12	2.6	64.0	0.0406	Folin [1]
8	2.51	65.0	0.0395	Klemperer [3]
19	2.98	76.2	0.0391	Thomas [6]
6	2.93	79.2	0.0370	Kocher [5]
9	2.25	61.4	0.0366	Graham and Poulton [7]
11	2.13	60.5	0.0352	Robison [8]
11	1.99	57.8	0.0344	Robison [8]
6	2.01	88.0	0.0319	Klercker [9]
—	1.84	58.0	0.0317	Sivén [10]
24	1.58	65.3	0.0242	Smith [11]
71	1.75	72.5	0.0241	Deuel and others [12]

NOTE: It is interesting to note that Petrén (*J. Biol. Chem.*, 1924, lxi, 355) in the clinical treatment of diabetic patients by means of diets very low in protein and carbohydrates and rich in fat, has obtained extremely low values for the day's excretion of urinary nitrogen. When expressed per kilogram of body weight these values range from 0.023 to 0.035 gms.

References:

1. Folin, O., *Amer. J. Physiol.*, 1905, xiii, 117.
2. Landergren, E., *Skand. Arch. Physiol.*, 1903, xiv, 112.
3. Klemperer, G., *Z. klin. Med.*, 1889, xvi, 550.
4. Roehl, W., *Deutsch. Arch. klin. Med.*, 1905, lxxxiii, 523.
5. Kocher, R. A., *ibid.*, 1914, cxv, 82.
6. Thomas, K., *Arch. Anat. Physiol., Physiol. Abt.*, 1909, 219.
7. Graham, G., and Poulton, E. P., *Quart. J. Med.*, 1912, vi, 82.
8. Robison, R., *Biochem. J.*, 1922, xvi, 131. *See also* Martin, C. J., and Robison, R., *ibid.*, 1922, xvi, 407.
9. Klercker, K. O., *Biochem. Z.*, 1907, iii, 45.
10. Sivén, V. O., *Skand. Arch. Physiol.*, 1900, x, 91.
11. Smith, M., *J. Biol. Chem.*, 1925, lxviii, 15.
12. Deuel, H. J., Sandiford, I., Sandiford, K., and Boothby, W. M., *J. Biol. Chem.*, 1928, lxxvi, 391.

In the experiment of Deuel and associates, the daily ration contained about 0.7 gram of nitrogen, but only 1500 to 1800 calories in the period (No. 5) in which the lowest level of nitrogen excretion was reached. The urine of the ninth day in this period, which followed an 8-day period of low-protein feeding, preceded by 54 days of protein-free feeding, contained 1.75 grams of nitrogen, equivalent to 0.0241 gram per kilogram of body weight, a value almost identical with that obtained by Smith. However, the distribution of nitrogen in the urine was different

in the conversion of arginine into creatine, one-fourth of its nitrogen is wasted. Aside from the doubtfulness of some of the assumptions made, the force of the evidence must be greatly discounted because the nitrogen intake was estimated from average analyses, not determined. Furthermore, it is questionable whether all the nitrogen absorbed would be excreted in the urine, except possibly in small part. It seems more probable that it would be largely if not entirely used in the replacement of the tissue constituents lost in the minimum endogenous catabolism.

TABLE 45. *Determinations of the Daily Excretion of Urinary Nitrogen by Animals on Low-Nitrogen or Nitrogen-Free Diets.*

Kind of Animal	Body Weight	Daily Urinary Nitrogen	Urinary N per Kgm. Body Weight	Investigator
	kgms.	gms.	gms.	
Pig	22.2	1.60	0.072	McCollum and Hoagland [1]
	14.3	0.96	0.067	McCollum and Hoagland [1]
	17.7	1.09	0.062	McCollum and Hoagland [1]
	19.5	1.09	0.056	McCollum and Steenbock [2]
	16.8	0.90	0.054	McCollum and Steenbock [2]
	25.0	1.32	0.053	Pfeiffer [3]
	38.1	2.00	0.052	Mitchell and Kick [4]
	10.9	0.54	0.050	McCollum and Steenbock [2]
	38.1	1.88	0.049	Mitchell and Kick [4]
	40.0	1.95	0.049	Mitchell and Kick [4]
	46.3	2.23	0.048	McCollum and Hoagland [1]
	38.5	1.83	0.047	McCollum and Steenbock [2]
	26.3	1.19	0.045	Pfeiffer [3]
	37.2	1.61	0.043	McCollum and Steenbock [2]
	68.1	2.65	0.039	McCollum and Steenbock [2]
	41.0	1.54	0.038	Morgen, et al. [5]
	74.9	2.61	0.035	McCollum and Steenbock [2]
Cattle	145	6.48	0.045	Steenbock, Nelson and Hart [6]
	385	16.32	0.042	Honcamp, Koudela and Muller [7]
	177	6.33	0.036	Hart, Humphrey and Morrison [8]
	440	16.40	0.035	Honcamp, Koudela and Muller [7]
	443	15	0.034	Copenhagen investigators [9]
	168	5.03	0.030	Hart, Humphrey and Morrison [8]
	485	14	0.029	Copenhagen investigators [9]
Sheep	47	3.39	0.072	Morgen, Beger and Westhauser [5]
	38	2.59	0.068	Morgen, Beger and Westhauser [5]
	35	2.37	0.068	Morgen, Beger and Westhauser [10]
	45	2.63	0.058	Morgen, Beger and Westhauser [10]
	43.5	2.39	0.055	Morgen, Beger and Westhauser [5]
	45	2.41	0.054	Morgen, Beger and Westhauser [10]
	35	1.81	0.052	Morgen, Beger and Westhauser [10]
	40	1.91	0.048	Morgen, Beger and Westhauser [10]
	42	1.84	0.044	Morgen, Beger and Westhauser [5]
	40	1.71	0.043	Morgen, Beger and Westhauser [10]
	54	2.02	0.038	Morgen, Beger and Westhauser [5]
	31.9	0.99	0.031	Völtz [11]
	33.1	1.03	0.031	Scheunert, Klein and Steuber [12]
	43.5	1.16	0.027	Scheunert, Klein and Steuber [12]
	44.1	1.05	0.024	Scheunert, Klein and Steuber [12]
Dog	12.7	2.20	0.173	Underhill and Goldschmidt [13]
	13.5	1.83	0.136	Underhill and Goldschmidt [13]
	12.2	1.61	0.132	Underhill and Goldschmidt [13]
	13.1	1.60	0.122	Underhill and Goldschmidt [13]
	18.5	1.71	0.092	Underhill and Goldschmidt [13]
Rabbit	1.76	0.249	0.141	Mendel and Rose [14]
	1.49	0.164	0.110	Serio [15]
	1.21	0.13	0.105	Myers and Fine [16]

References:

1. McCollum, E. V., and Hoagland, D. R., *J. Biol. Chem.*, 1913, xvi, 305.
2. McCollum, E. V., and Steenbock, H., *Wisc. Agr. Exp. Sta. Res. Bull.* 21, 1912.
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5. Morgen, A., Beger, C., and Westhauser, F., *Landw. Vers-Sta.*, 1914, lxxxv, 1.
6. Steenbock, H., Nelson, V. E., and Hart, E. B., *Wisc. Agr. Exp. Sta. Res. Bull.* 36, 1915.
7. Honcamp, F., Koudela, St., and Muller, E., *Biochem. Z.*, 1923, cxliii, 111.
8. Hart, E. B., Humphrey, G. C., and Morrison, F. B., *J. Biol. Chem.*, 1912, xiii, 133.
9. Copenhagen Laboratory, cited by Buschmann, A., *Landw. Vers.-Sta.*, 1907, ci, 16.
10. Morgen, A., Beger, C., and Westhauser, F., *Landw. Vers.-Sta.*, 1911, lxxv, 265.
11. Völtz, W., *Biochem. Z.*, 1920, cii, 151.
12. Scheunert, A., Klein, W., and Steuber, M., *Biochem. Z.*, 1922, cxxxiii, 137.
13. Underhill, F. P., and Goldschmidt, S., *J. Biol. Chem.*, 1913, xv, 341.
14. Mendel, L. B., and Rose, W. C., *J. Biol. Chem.*, 1911, x, 226.
15. Serio, F., *Biochem. Z.*, 1923, cxlii, 440.
16. Meyers, V. C., and Fine, M. S., *J. Biol. Chem.*, 1913, xv, 305.

in this experiment, 43 per cent being present as urea, 10 per cent as ammonia, 32 per cent as creatinine, and 8 per cent as uric acid.

A number of more or less successful attempts to measure the minimum endogenous catabolism of the lower animals have been reported in the literature. The results of these attempts are recorded in Table 45. The pig has proven to be an excellent subject in such work, consuming regularly such amounts of nitrogen-free rations as to maintain his weight for several weeks. Hence the results given for this animal are highly significant as measures of endogenous catabolism. The values for the pig bear a remarkable similarity to those for humans. In general they lend support to the conclusion that the minimum endogenous urinary nitrogen per kilogram of body weight decreases as the size (age?) of the pig increases, though evidently other factors exert a large influence also.

The values for the calf and the sheep are of less significance than those for the pig because of the difficulty of inducing these animals to consume nitrogen-free rations in adequate amounts for any considerable period of time. They are, however, of the same order of magnitude as the values for pigs. The minimum endogenous urinary nitrogen of dogs and rabbits per unit of weight seems to be considerably higher than that for the other animals. The same is true of the rat. In the course of the many determinations of the biological value of proteins undertaken by the Division of Animal Nutrition of the University of Illinois, the minimum endogenous urinary nitrogen was determined on a large number of rats by the feeding either of nitrogen-free rations or, more recently, of rations containing approximately 0.75 per cent of whole egg nitrogen, which is utilized at this level of feeding to an extent close to 100 per cent. A summary of the values thus obtained on 425 rats is given in Table 46, the results being arranged in the order of weight. As was true

TABLE 46. *The Endogenous Nitrogen of Rats of Different Weight.*

Body Weight	No. of Rats in Group	Average Endogenous Nitrogen per Kgm. Body Weight
gms.		gms.
40- 60	27	0.219
60- 80	95	0.236
80-100	66	0.227
100-120	83	0.194
120-140	51	0.166
140-160	32	0.195
160-180	37	0.199
180-200	21	0.174
200-220	10	0.165
220-240	3	0.110

of the pig, the endogenous nitrogen per unit of weight tends definitely to decrease with increasing weight and age, although many other factors are evidently concerned in determining this value. In the rat particularly, the minimum endogenous metabolism appears to vary widely from animal to animal, due to hereditary factors or to unknown experimental conditions.

THE CREATININE COEFFICIENT

While the total minimum endogenous catabolism can be determined only by nitrogen-free feeding, it is possible to obtain some information concerning the *relative* endogenous catabolism of different individuals or concerning the effect of different experimental conditions upon the minimum endogenous catabolism by determining the daily excretion of creatinine. On creatine-free diets, the urinary creatinine appears to be entirely derived from the minimum endogenous catabolism. The interpretation of the excretion of creatinine in terms of the endogenous catabolism obviously implies that creatinine nitrogen accounts for a fairly constant fraction of the total endogenous nitrogen. This implied constancy, however, is hardly justified by available data. Smith has shown that in recorded experiments on human subjects maintained on low-nitrogen or nitrogen-free diets (Table 44) the creatinine nitrogen in per cent of the total urinary nitrogen varies from 11.5 to 35.4; however, considering only those experiments in which the endogenous catabolism appears to have been closely approximated, the range is only from about 20 to 35, with one exception, *i.e.,* 14.0 per cent. McCollum and his associates [46, 65] have found much the same for swine: the average percentage is given as 18.5, but considerable variation was noted from this figure (9.4 to 22.0). In the rat,[66] creatinine accounts for only about 8 per cent of the total endogenous urinary nitrogen, and again considerable variations from the average occasionally occur. It seems impossible to account definitely for these variations, though McCollum has suggested that they may be related to vitality. In his experience, pigs of low vitality will generally exhibit a low percentage of creatinine nitrogen on starch diets.

The creatinine excretion has been expressed also with reference to body weight. This is Shaffer's so-called "creatinine coefficient," *i.e.,* the number of milligrams of creatinine (or creatinine nitrogen) excreted daily per kilogram of body weight. In summarizing the values assumed by this coefficient among human subjects, Hunter,[25] says:

[65] McCollum, E. V., and Steenbock, H., *Wisc. Agr. Exp. Sta., Res. Bul.* 21, 1912.
[66] Mitchell, H. H., and Carman, G. G., *J. Biol. Chem.,* 1926, lxviii, 183. Also unpublished data.

"This coefficient, expressed in terms of creatinine, varies in 'strictly normal' human adults of the male sex from 18 to 32, though possibly the lower limit of normality should be reduced to 15. In normal women the figures have the decidedly lower range of 9 to 26, averaging only 15.6; it is probable, as Shaffer thought, that this is not an effect of sex itself, for individual women whose muscles have been developed by gymnastic training have coefficients comparable with those of men. Infants and children have lower coefficients still: 6.7 to 10 at 10 to 14 days, and 9 to 17 at 5 to 13 years. The considerable range of the figures in every group shows that the relation between creatinine output and total body weight is not one of strict proportionality." Apparently the tone of the muscles, or their predominance in metabolism, is intimately related to the creatinine coefficient of the individual, a conclusion that is understandable since the muscles contain about 98 per cent of the body creatine, from which the urinary creatinine appears to be derived.

The lower animals excreting about the same amount of endogenous urinary nitrogen per unit of weight as man (see Table 45), possess creatinine coefficients closely similar to those found for human subjects. In the experiment of McCollum and associates, eleven pigs ranging in weight from 11 to 75 kgms., possessed an average coefficient of 23, the range being from 17 to 26. Forbes and associates [67] obtained an average creatinine coefficient of 24 for five pigs. In these experiments there is a distinct indication of an increase in the coefficient as the pigs increased in size. In an investigation of the composition of the urine of two fasting steers, Carpenter [68] found the creatinine coefficient of one to vary between 20 and 23, and that of the other between 21 and 27. Underhill and Goldschmidt [69] obtained creatinine coefficients for four dogs ranging from 19 to 32 and averaging 28.

On the other hand, those animals possessing a greater endogenous catabolism per unit of weight than men also show greater creatinine coefficients. Myers and Fine [70] obtained an average coefficient of 40 for four rabbits (range 34 to 48), while Mendel and Rose [71] obtained a coefficient of 46 on one rabbit. In the case of rats, Rose and Cook [72] obtained creatinine coefficients ranging from 46 to 67 and averaging 56 from twelve rats varying in weight from 70 to 200 grams. Morgan and Osburn [73] have reported creatinine coefficients of 35 to 38 for growing

[67] Forbes, E. B., Beegle, F. M., Fritz., C. M., and Mensching, J. E., *Ohio Agr. Exper. Sta. Bul.* 271, 1914.
[68] Carpenter, T. M.. *Proc. Nat. Acad. Sci.*, 1925, xi, 155.
[69] Underhill, F. P., and Goldschmidt, S., *J. Biol. Chem.*, 1913, xv, 341.
[70] Meyers, V. C., and Fine, M. S., *J. Biol. Chem.*, 1913, xv, 305.
[71] Mendel, L. B., and Rose, W. C., *J. Biol. Chem.*, 1911, x, 226.
[72] Rose, W. C., and Cook, K. G., *J. Biol. Chem.*, 1925, lxiv, 325.
[73] Morgan, A. F., and Osburn, D. F., *J. Biol. Chem.*, 1925, lxvi, 573.

and adult rats, while Folin and Morris [74] found an average value of 40 for adult rats and 27 for young rats. The results of Mitchell and Carman [66] indicate creatinine coefficients ranging from 54 to 81 for a number of growing rats, though in unpublished work coefficients as low as 40 have been obtained. Apparently in both rabbits and rats the minimum endogenous metabolism is much more intense than in larger animals.

THE EFFECT OF WORK UPON THE ENDOGENOUS CATABOLISM

Since the days of Liebig, the relation of muscular contraction to protein metabolism has been shown to be of less and less importance. The classic experiments of Fick and Wislicenus, which have been abundantly confirmed, showed that muscular work could be performed at the expense of carbohydrates and fats, while the trend of modern investigation into the chemical and calorimetric phases of muscular contraction [75] is toward the view that carbohydrate is the immediate source of muscular energy. The entire series of events occurring during and subsequent to a muscular contraction can be described, apparently, without reference to protein, amino acids, or the nitrogenous extractives, with the exception of creatine, which apparently is not destroyed in the process (see footnote to p. 483). Furthermore, the so-called fatigue products of muscles, in so far as available information indicates, are neither nitrogenous in character nor necessarily related to protein metabolism.[76] It is true that investigations are on record indicating changes in the content of purines [77] and of creatine [78, 56] in muscle as the result of activity, but the relation of these changes to the activity itself is obscure. The relation is probably indirect, and the function of the non-protein nitrogenous constituents of muscle relative to contraction is quite possibly regulatory only. Functioning in this way it is conceivable that they may not be consumable during muscular activity.

To many investigators it has seemed almost axiomatic that muscle tissue must undergo disintegration as the result of contractile activity. And when experimental investigation has failed to indicate any such disintegration, or at least any considerable disintegration, it has been considered necessary to assume that most of the nitrogen thus degraded escapes excretion in the urine by some process of re-utilization in the

[74] Folin, O., and Morris, J. L., *J. Biol. Chem.*, 1913, xiv, 509.
[75] Hill, A. V., "Muscular Activity," Baltimore, 1926.
[76] Scott, E. L., *Public Health Reports*, 1918, xxxiii, 605.
[77] Burian, R., *Z. physiol. Chem.*, 1904-05, xliii, 532; Macleod, J. J. R., *Z. physiol. Chem.*, 1899, xxviii, 535.
[78] Brown, G., and Cathcart, E. P., *Biochem. J.*, 1909, iv, 420; Pekelharing, C. A., and van Hoogenhuyze, C. J. C., *Z. physiol. Chem.*, 1909, lxiv, 262; Uyeno, K., and Mitsuda, T., *J. Physiol.*, 1923, lvii, 313; Schlossmann, H., *Z. physiol. Chem.*, 1924, cxxxix, 87; *Arch. exp. Path. Pharm.*, 1925, cv, p. xiv.

tissues,[79] a hypothesis apparently beyond the scope of experimental inquiry.

It seems that this view of the necessary wastage of muscle tissue during activity has resulted from the analogy so often drawn between the mechanical motor and the animal motor. In the early history of physiology this analogy has served an admirable purpose, and in pedagogy it is still extremely useful. But, like many other analogies, it is only partially true, and if pushed too far it will confuse rather than enlighten. It seems apparent that one phase of motor activity to which the analogy has an extremely doubtful application, if it applies at all, is the wearability of the motor. The mechanical motor undoubtedly wears out at a rate that bears a close relation to the amount or the intensity of the work performed. But this wearing out is due mainly to the friction of moving parts; that is, to a factor that has no *known* counterpart at least in the animal body. Nevertheless, the opinion prevails in many quarters and is frequently expressed in print, that the animal motor also must wear out, and since the substance of the animal motor is largely protein, the conclusion has seemed reasonable, if not inevitable, that the protein catabolism must be increased as the result of muscular work. Rubner's term for the maintenance requirement of protein, the "wear-and-tear quota," obviously is based upon this analogy of the mechanical motor and obviously implies that the catabolism of tissue protein will increase with increased motor activity. The term is an unfortunate one, particularly since it has attained a wide currency.

Since as early as 1855, the influence of work on protein metabolism has been a favorite subject of inquiry, and a considerable number of experiments have been reported in the literature. However, the results secured have been variable and at the present writing opinion is divided as to whether work increases the catabolism of tissue proteins or is without appreciable effect. From time to time these experiments have been reviewed, but it appears to be difficult to reconcile them with any definite conclusion. In 1909 they were reviewed by Tigerstedt,[80] who was not convinced that there is an increase of nitrogen metabolism resulting from muscular work. On the other hand, they were reviewed by Magnus-Levy [81] at about the same time (1907), and the conclusion was reached that it is probable that such an effect occurs. In 1917 Lusk [82] concluded from a study of the same literature not only that "muscular work does not increase protein metabolism," but also that "the character of the

[79] Cathcart, E. P., "The Physiology of Protein Metabolism," London, 1921, p. 134.
[80] Tigerstedt, R., "Handbuch der Physiologie des Menschen," by W. Nagel, pp. 441-451, 1909.
[81] Magnus-Levy, A., "Metabolism and Practical Medicine," vol. I, by C. von Noorden, Amer. trans., pp. 352-355, 1907.
[82] Lusk, G., "The Science of Nutrition," 3rd ed., Philadelphia, 1917, p. 316.

protein metabolism is unchanged by muscular activity." However, in 1925 Cathcart [83] again reviewed the literature, which, in conjunction with recent experiments of his own, induced him to state that, in spite of this very definite conclusion of Lusk, "the accumulated evidence seems to me to point in no unmistakable fashion to the opposite conclusion, that muscle activity does increase, if often only in small degree, the metabolism of protein," although "there is no possible ground for the view that protein is *the* source of muscular energy."

It would be far beyond the scope of this monograph to inquire into the many reasons why critics weigh essentially the same evidence with such discordant results. It appears to be due in part to different criteria as to what constitutes competent experimental evidence. Thus, Atwater and Sherman [84] insist that the diet during the working period should be increased in its energy content to allow for the increased energy requirement; otherwise, an increase in the protein catabolism may mean simply that tissue protein is being destroyed as a source of muscular energy, rather than as the result of an inevitable wear on the muscular tissues. Cathcart, however, is not so discriminating, and does not disregard or even discount experiments in which no such assurance is given. Again, the results of some experiments are considered *in toto* by some, and only in part by others. Shaffer's [85] well-known experiment offers a case in point. Considering the average urinary excretions of rest and work periods, Shaffer and Lusk both conclude that work has had no effect on the nitrogenous metabolism. Cathcart, however, notes a marked increase in urinary nitrogen on the fourth work day and, without reference to the rest periods at all, implies that this is a result of the work performed. Cathcart's recent experiments,[86] although very carefully controlled and undivided in their obvious interpretation, seem no more convincing than the much earlier experiments of Wait [87] at the University of Tennessee, which yielded entirely negative results. These experiments of Wait are not considered in Cathcart's review.

The maze of conflicting experimental results relative to the effect of work on protein metabolism suggests that a precise definition of the real problem, followed by a consideration of the proper experimental conditions to impose, might be of value in deciding as to which of the recorded experiments are capable of the most exact interpretation. Since there is now no question of the availability of protein as a source of energy for muscular work, the real problem becomes that of determin-

[83] Cathcart, E., P., *Physiol. Revs.*, 1925, v, 225.
[84] Atwater, A. O., and Sherman, H. C., *U. S. Dept. Agr., Office Exp. Sta., Bull.* 98, 1901.
[85] Shaffer, P. A., *Amer. J. Physiol.*, 1908 xxii, 445.
[86] Cathcart, E. P., and Burnett, W. A., *Proc. Roy. Soc. London*, Series B, 1926, xcix, 405.
[87] Wait, C. E., *U. S. Dept. Agr., Office Exp. Sta. Bull.* 117, 1902.

ing whether or not there is an *inevitable* disintegration of muscle protein as a result of muscular activity.

This definition of the problem imposes certain necessary conditions upon any experimental method of attack. In the first place, there should be an adequate intake of energy during the working periods, otherwise an increase in urinary nitrogen may mean a destruction of tissue protein merely to serve the unnecessary rôle of a source of energy. If no evidence is obtained of an increase in tissue destruction as the result of work, this in itself would seem to be satisfactory evidence that the energy intake was adequate. On the contrary, if the experimental results indicate an increased destruction of tissue, the investigator himself must assume the burden of the proof that the energy intake was adequate and hence that the increased tissue catabolism is an *inevitable* consequence of muscular activity. Thus, the significance of negative results in the investigation of this problem would seem to be much more easily established than the significance of positive results.

The only known method of investigating the extent of the destruction of tissue protein under any experimental conditions is to determine the nitrogen content of the urine on a nitrogen-free diet of adequate energy value, and even under these conditions the contribution of the non-protein nitrogenous constituents to the urinary nitrogen cannot be determined. It would seem to be imposible, therefore, to rule out the rôle of the latter constituents in the phenomena under investigation. However, it is equally clear that if protein is included in the experimental diet, at least in quantities above the absolute minimum required to replace endogenous losses of nitrogen, the significance of the urinary nitrogen is difficult if not impossible to establish definitely. Under such conditions the urine contains the products of an exogenous protein metabolism, the intensity of which for a given protein intake will vary with the intensity of the prevailing anabolic processes and with the extent to which the protein is utilized in anabolism. Hence, even though the protein intake is kept constant in rest and work periods, the output of urinary nitrogen might be affected if muscular activity alters either the intensity of the anabolic processes or the extent to which the dietary protein is utilized in anabolism. It is not inconceivable that an increase in muscular activity may do both, and hence a slight increase in the day's urinary nitrogen in work as compared with rest periods may bear no relation to the intensity of the minimum endogenous metabolism.

On the other hand, if the energy intake is adequate, an increased catabolism of muscle due to increased activity may be entirely obscured if the protein intake exceeds the requirements, since dietary amino acids

may be diverted from catabolic to anabolic processes with no effect on the output of urinary nitrogen.

Returning now to past experiments on the effect of work on protein metabolism, as reviewed, for example, by Cathcart, the increases in the excretion of urinary nitrogen as a result of work, so frequently noted, are in most cases not accompanied by a demonstration that the intake of energy was adequate; in fact, in a considerable number of cases a suspicion that the contrary was true is almost inevitable. Furthermore, in the large majority of experiments, the protein intake was much above the minimum requirement, and in some cases was excessive. The precise interpretation of the positive results obtained with such diets seems impossible. They may have resulted from a depression of the anabolic processes involving dietary protein, or from a depression of its biological utilization. While it is true that work tends to muscular hypertrophy, it seems more probable that this increase in anabolism is an after effect of work rather than a contemporary effect, since the immediate result of muscular contraction is undoubtedly catabolic. Even in the adult, some tissues are continually growing. If this growth is inhibited during muscular activity on a protein-containing dietary, the urinary nitrogen will be increased by a corresponding amount. The effect of muscular activity on the utilization of absorbed protein in metabolism is entirely unknown, but it is not inconceivable that a depression of utilization, with a corresponding increase in urinary nitrogen (or sulfur) may result. It might even be considered a matter for surprise if the great acceleration in the catabolic processes of the body during muscular work did not conscript some of the dietary amino acids that otherwise would be used for the growth of hair or other tissues, or for the replenishment of the digestive glands, or for the replacement of the nitrogenous losses resulting from the minimum endogenous catabolism. Entirely aside from such admittedly hypothetical considerations as these, it would seem a futile undertaking to detect the effect of muscular work upon the rate of tissue catabolism that is measured normally, in the human subject, by the excretion of 1.5 to 3 grams of nitrogen in the urine daily, by imposing upon this an exogenous protein catabolism yielding 5 or 10 times as much urinary nitrogen.

Such considerations as these lead to the conclusion that the characteristic effect of work on tissue catabolism should be investigated under conditions such that the exogenous catabolism of nitrogen is entirely eliminated or is reduced to an insignificant minimum. This can be accomplished by the feeding of a very low-nitrogen diet, or, if possible, a nitrogen-free diet. Furthermore, the experiment proper should

be preceded by a feeding period of sufficient length to remove the "deposit protein" from the tissues, since this stored nitrogen will tend to vitiate the results of the experiment in the same way as would dietary nitrogen.

On protein-containing but creatine-free dietaries, the excretion of creatinine nitrogen is generally considered to be proportional to the total endogenous nitrogen. Hence, the effect of work upon the creatinine excretion is taken to indicate the effect upon muscle endogenous catabolism. While the reasoning upon which this conclusion is based is not fortified throughout by adequate experimental data, it may be considered justifiable if not entirely convincing.

Of all the experiments that have been reviewed so thoroughly from time to time, there appear to be only two in which the diet was approximately nitrogen-free and at the same time was approximately adequate in energy value, and in which the period of nitrogen-free feeding was sufficiently extended so that the urinary nitrogen had approximated the endogenous level. These are the experiments of Thomas [88] reported in 1910, and those of Kocher [89] reported in 1914.

Thomas reduced his output of urinary nitrogen to approximately the endogenous level by subsisting on a diet of pure sugar. Following a fore-period of four days at this level was a work period of three days during which work on an ergostat was performed amounting to 120,000 kgm. meters. The experiment was concluded by an after period of three days on the same diet. The daily excretions of urinary nitrogen were as follows: fore-period—3.05, 2.73, 3.22 and 2.85 grams; work period—2.47, 2.90, and 2.97 grams; and after period—2.71, 2.22, and 2.31 grams. Judged by a comparison of the average excretions only, the work had no effect on the excretion of urinary nitrogen, the averages being, respectively, 2.96, 2.78 and 2.41 grams. If it is considered that in the latter period only was the endogenous level of tissue catabolism reached, Thomas concludes that the work might have effected a slight increase in muscle catabolism. However, if the endogenous catabolism were not attained until the last few days of the experiment, the increased excretion of nitrogen in the work period above this level would appear to be due more probably to the elimination of "deposit protein" than to an effect of work. This experiment of Thomas, therefore, does not support the conclusion that muscular work increases the endogenous muscular catabolism. It is to be noted that definite proof of the adequacy of the diet in energy value during the work period by a comparison of intake

[88] Thomas, K., *Arch. Physiol., Supl. Band,* 1910, 249.
[89] Kocher, R. A., *Deutsch. Arch. klin. Med.,* 1914, cxv, 82.

with outgo of energy, is not required when the urinary nitrogen shows no increase in this period.*

Kocher's experiment involved two subjects and a study of the nitrogenous constituents in the urine. The diet used contained a minimal amount of nitrogen (1.01 gram per day) derived entirely from cream and contained over 5000 cals. daily. In each case there was but one work day, during which the subject walked 60 kilometers (about 37.8 miles) in 10 hours. The results for this day and for the three preceding and the three following days are given in Table 47.

TABLE 47. *Kocher's Results on the Effect of Work on the Endogenous Catabolism.*

Exper. Day	Body Weight	Urine Volume	Total Urinary Nitrogen	Ammonia Nitrogen	Creatinine Nitrogen	Uric Acid Nitrogen	Total Sulfur
	kgms.	cc.	gms.	gms.	gms.	gms.	gms.
			Subject R.A.K.				
4	...	1800	4.05	0.402	0.603	....	0.268
5	79.2	1660	2.92	0.453	0.692	....	0.265
6	79.2	1900	2.93	0.478	0.693	....	0.264
7[a]	...	**1195**	**3.89**	**0.323**	**0.915**	....	**0.335**
8	79	1395	4.69	0.337	0.773	....	0.330
9	79	1665	4.29	0.372	0.781	....	0.257
10	79.2	1440	3.07	0.367	0.684	....	0.249
			Subject J.G.F.				
3	...	3005	3.35	0.495	0.762	0.100	0.214
4	70.4	1850	2.89	0.460	0.742	0.064	0.186
5	...	2980	3.24	0.490	0.775	0.132	0.184
6[a]	...	**2500**	**3.77**	**0.345**	**0.825**	**0.135**	**0.199**
7	69.9	1595	3.71	0.284	0.758	0.107	0.175
8	71.0	1850	2.89	0.368	0.756	0.123	0.204
9[b]	70.9	2330	3.35	0.036	0.758	0.183	0.198

[a] Work day—walking 60 kilometers in 10 hrs.
[b] Took 30 gms. of $NaHCO_3$.

It will be noted that with both subjects the total urinary nitrogen and the creatinine nitrogen increased on the day of work, and that the increased excretion continued for the next day or two. The effect is much more noticeable in the first subject than in the second, a fact possibly correlated with the much lower creatinine coefficient of the first subject. Unfortunately there is not sufficient assurance that the calorie intake of the work days was adequate. On the basis of average values for the energy requirements at rest and during horizontal walking,

* Since the above was written, evidence confirming the results of Thomas have been obtained. Mitchell and Kruger (*J. Biol. Chem.*, 1928, lxxvi, 55) in experiments involving the feeding of rats on nitrogen-free rations, have obtained no evidence of an increase in endogenous nitrogen catabolism as a result of either static or dynamic work, provided the food intake was adequate for the maintenance of weight. This was true whether the ration was predominantly carbohydrate or predominantly fat and containing only traces of carbohydrate. In work on dogs, Chambers and Milhorat (*J. Biol. Chem.*, 1928, lxxvii, 603) showed that the urinary output of nitrogen in fasting was greatly increased by work, but that this increase could be entirely obviated by the administration of carbohydrates.

Kocher estimates that the energy intake of Subject R.A.K. was about 4.0 per cent in excess of the requirements on the day of work, while with Subject J.G.F. the estimated excess was 4.6 per cent. However, the estimates of energy requirements might conceivably be in error by much more than this. Hence, the experiments do not demonstrate that an increase in endogenous catabolism is an inevitable consequence of muscular work; in other words, that there is an inevitable wear on the muscle machine.

In further experiments on the same subjects, using a high-protein high-fat diet, the urinary nitrogen showed no increase as a result of work when the calorie intake was high, but a distinct increase when the calorie intake was deliberately lowered so as to be obviously insufficient. With the other subject, on a high-protein diet, even when the calorie intake was obviously insufficient, the urinary nitrogen did not increase as a result of work. Of more significance than the total urinary nitrogen on a high-protein diet are the values for the creatinine excretion. These appeared to be unaffected by work, except for a slight increase on the day of work in one of the experiments in which the energy intake was deliberately made insufficient.

The experiments of Cathcart and Burnett [86] also indicate a slight effect of work upon the creatinine excretion. The diets used in the different series of experiments varied in nitrogen content, but were all estimated to contain 2900 calories. Their specific dynamic effect probably approximated 200 cals. In each series the diet was constant in the pre-work periods of 4 days, the work periods of six days, and the post-work periods of 4 days. The work was performed for one hour daily on a hand-level ergometer and was equivalent to 25,000 kgm.-meters (equivalent to 58.6 cals.). Depending upon whether the efficiency is taken at 20 or 25 per cent, the heat output due to this quantity of work may be taken as 295 or 236 cals. The subject weighed 79 kgms., and, with a surface area of 1.99 sq. meters, would have a basal requirement of 1920 cals. If the estimate of the energy intake is accurate, and the other activities of the working days did not exceed 500 cals., the energy intake may be considered as adequate. During the twelve months of experimental feeding the subject gained 1.6 kgms., but in seven of the eleven working periods, a slight loss in weight occurred. The adequacy of the energy intake for the entire experiment cannot be doubted, but it is unfortunately not equally clear that the diet was adequate during the working days. The average data of the four series of experiments are summarized by Cathcart and Burnett as follows:

TABLE 48. *The Average Data of Daily Urinary Excretion from the Experiments of Cathcart and Burnett.*

(*All weights in grams.*)

	Series I			Series II		
	Pre-work	Work	Post-work	Pre-work	Work	Post-work
Total nitrogen	8.78	10.01	9.05	17.31	19.96	17.76
Ammonia nitrogen	0.426	0.468	0.474	0.706	0.811	0.686
Uric acid nitrogen	0.189	0.198	0.155	0.220	0.229	0.205
Creatinine nitrogen	0.543	0.562	0.552	0.603	0.633	0.620
Total sulfur	0.812	0.853	0.826	1.151	1.223	1.194
Total P_2O_5	2.20	2.33	2.14	3.52	3.47	3.38

	Series III			Series IV		
	Pre-work	Work	Post-work	Pre-work	Work	Post-work
Total nitrogen	17.40	18.23	17.34	14.30	15.44	16.34
Ammonia nitrogen	0.656	0.673	0.644	0.626	0.654	0.629
Uric acid nitrogen	0.242	0.223	0.229	0.217	0.226	0.227
Creatinine nitrogen	0.642	0.682	0.669	0.667	0.710	0.679
Total sulfur	1.115	1.140	1.118	1.000	1.059	1.154
Total P_2O_5	3.68	3.75	3.61	3.53	3.19	3.25

Although it is difficult to interpret the figures for total urinary nitrogen and sulfur with reference to an effect of work, for reasons fully explained above, the slight increases in creatinine nitrogen during the work periods were sufficiently consistent to indicate a direct or indirect effect of work upon the endogenous catabolism of muscle.

While the experiments of Kocher and of Cathcart and Burnett * thus indicated an increased excretion of creatinine simultaneous with the performance of fatiguing muscular work, other investigators [85, 90] have obtained no such increases. If the creatinine excretion is followed at intervals as short as two hours, the output during a short period of work has been found by Shulz [91] to be invariably increased, often to a large extent; however, in some later period of the day, generally the following period, the creatinine output is with equal regularity decreased, so that the total day's output of creatinine is not appreciably affected by the work performed. Hartmann [92] found much the same fluctuations in the uric acid and phosphoric acid content of 2-hour urine samples, and suggests that such variations are not due to variations in the production of these compounds, but in the excretory activity of the kidneys as a result of muscular work.† That the excretory activity of

* A more recent experiment by R. C. Garry (*J. Physiol.*, 1926-27, lxxii, 364) possesses much the same significance as that of Cathcart and Burnett for work on an ergometer, but no clear effect of static effort on the excretion of total nitrogen or of creatine was reported.

[90] Hoogenhuyze, C. J. C. van, and Verploegh, H., *Z. physiol. Chem.*, 1905, xlvi, 415.

[91] Schulz, W., *Arch. ges. Physiol.*, 1921, clxxxvi, 126.

[92] Hartmann, C., *Arch. ges. Physiol.*, 1924, cciv, 613.

† This explanation is supported by the experimental finds of Rakestraw (*J. Biol. Chem.*, 1921, xlvii, 565), as well as of Levine, Gordon and Derick (*J. Amer. Med. Assoc.*, 1924, lxxxii, 1778), who have noted consistent increases in the uric acid concentration of the blood during work, possibly due to diminished kidney function.

the kidney is affected by muscular work has been clearly shown by Wilson and associates.[93]

While negative results in experiments concerned with the effect of work upon the minimum endogenous catabolism are undoubtedly more significant than positive results, they cannot be considered as *refuting* positive results. Hence, from the few data of effective experimental investigations at present available it appears that an increased breakdown of body tissue is not an *inevitable* consequence of muscular work, but that it may occur, even though the diet is sufficient in energy. The conditions under which such an increase occurs are unknown. Possibly if the general body temperature, or the temperature of the working muscles, increases above a certain point, the endogenous catabolic processes, whatever they may be, are speeded up, with a resulting increase in the output of endogenous metabolites in the urine. Experiments by Myers and Volovic [31] and by Linser and Schmid [94] show that increases in body temperature artificially induced in healthy subjects may increase the endogenous catabolism.

The interesting results of Lee and Tashiro [95] indicating an increased production of ammonia in excised muscles during contraction may have a bearing on the general problem of the effect of work on the endogenous catabolism. On the other hand, this production of ammonia may be due to the abnormal condition of the excised muscle, or to the lack of readily available sources of energy, or to anoxemia.[96]

[93] Wilson, D. W., Long, W. L., Thompson, H. C., and Thurlow, S., *Proc. Soc. Exper. Biol. Med.*, 1923-24, xxi, 425.
[94] Linser, P., and Schmid, J., *Deutsch. Arch. klin. Med.*, 1903-4, lxxix, 514.
[95] Lee, O. P., and Tashiro, S., *Amer. J. Physiol.*, 1922, lxi, 244.
[96] Brunquist, E. H., Schneller, E. J., and Loevenhart, A. S., *J. Biol. Chem.*, 1924, lxii, 93.

CHAPTER X

THE NUTRITIVE VALUES OF PROTEINS AND THE PROTEIN VALUES OF FOODS IN NUTRITION

The peculiar functions of amino acids in animal nutrition that no other nutrients can serve relate to the replenishment and the enlargement of the nitrogenous components of the tissues and to the elaboration of the nitrogenous constituents of the products of reproductive and mammary organs. These nitrogenous substances are largely protein in nature, though each animal tissue and secretion contains non-protein nitrogenous compounds in greater or less abundance.

In so far as the amino acids are used in the production of nitrogenous substances of simple molecular structure, the requirement for amino acids is "particulate" in character; that is, each amino acid is an obligate or a facultative precursor of certain definite compounds, in the construction of which other amino acids are not (or need not be) used. Hence, the utilization of any one amino acid in this category of functions is not limited, in any direct fashion at least, by the absence or the relative deficiency of any other amino acid.

However, in the building-up of the protein molecule, conditions are obviously different. Here the requirement for amino acids is "aggregate" in nature, in the sense that a certain assortment of amino acids—at least of the indispensable amino acids—must be present in the tissues before any headway at all in protein synthesis can be made. Here the absence of one of the indispensable amino acids will block all synthesis effectively, and a relative deficiency in such an amino acid will permit only a subnormal rate of synthesis.

Hence, particularly in protein synthesis, the proportion in which the amino acids reach the tissues is an important determining factor in their functioning. In practical nutrition these proportions are determined by the character of the diet consumed, in particular by the character of the protein component of the diet. It becomes a matter of importance, therefore, to measure the efficiency with which different proteins and protein mixtures serve the body's requirements for amino acids.

THE BIOLOGICAL EVALUATION OF PROTEIN

The ultimate limit to the value of any protein or food or ration in covering the protein requirement of animals is the amino acid content. Hence, a quantitative study of the composition of the food material with respect to its constituent amino acids will reveal what the food contains, if not what the animal can assimilate of it.

In digesting proteins and protein-containing foods, the animal body suffers two types of losses of amino acids, one represented in the undigested and unabsorbed fraction of the food, and the other in the loss of nitrogenous substances from the body itself through the intestinal epithelium, *i.e.*, the so-called metabolic materials in the feces. The latter loss is probably unrelated to the kind or amount of protein ingested, being apparently determined largely by the amount of solid material eaten and to some extent by its indigestibility.[1] Nothing is known of its amino acid make-up or antecedents.

The amino acid loss in the undigested food residues, however, would be related to the composition of the protein consumed. However, it is equally probable that the proportions existing among the amino acids found in the food residues would not be the same as the proportions found in the food itself, and may be considerably different, since, as Abderhalden has shown, certain specific peptides are much more resistant to ereptic action than others. Therefore, the digested fraction of protein may be quite different in its amino acid yield from the protein consumed.

The wastage of food protein in digestion seems to be largely unrelated to its chemical structure,[2] at least to the proportions existing among the amino acids of which it is composed. On the other hand, the wastage of food protein in metabolism in covering the protein requirements of the body is generally ascribed entirely to its chemical structure. Except for those amino acids incidentally drawn into the oxidative catabolism of the tissues in accordance with the laws of mass action, the deamination of amino acids is assumed to depend upon the relation existing between the composite demands of the body and the assortment of amino acids coming to the tissues from the intestinal tract. The deficiency of the latter in some one indispensable amino acid will limit the utilization of all of the others, in so far as the "aggregate" requirements are concerned. Since the body's demands for amino acids are assumed to involve rather constant proportions among the different individual acids, the idea has gained currency that each protein, or each

[1] Mitchell, H. H., "The determination of the protein requirements of animals and of the protein values of farm feeds and rations." *Bull. National Res. Council,* 1926, xi, Part 1, No. 55.
[2] Mitchell, H. H., *Physiol. Revs.,* 1924, iv, 424.

definite protein mixture possesses a definite value in nutrition. In other words, the "biological value" of each protein or protein mixture, using the term in the technical sense given to it by Thomas [3] as the maximum percentage of the absorbed protein usable in anabolism, is assumed to be constant.[4] Consequently any method giving variable biological values is assumed *ipso facto* to be at fault.[5]

However, even though it is obvious that the chemical constitution of a protein or mixture of proteins rigidly sets an upper limit to its utilization in anabolism, it is not improbable that physiological factors may operate to vary its percentage utilization in definite ways according to the conditions of feeding or the physiological condition of the animal. Considering first the oxidative processes of the body, it appears to be quite often assumed or implied [6] that a growing animal, for example, will utilize the digestible protein in its ration to a constant extent, characteristic of the protein fed, up to the limit of its demands for this nutrient; when its protein demands are completely satisfied, then and then only are the dietary amino acids available primarily as sources of energy. But this is quite an artificial conception, conveniently simple, it is true, but otherwise unacceptable.

It appears more logical to suppose that at all times dietary amino acids are drawn into the oxidative reactions of the tissues in proportion to their relative concentration in the intracellular fluids with reference to the other organic nutrients and to the post-absorptive level, and also in proportion to their relative oxidizability as compared with glucose, fats, lipoids, etc. Hence the extent to which they are so used will decrease with increasing proportions of non-nitrogenous nutrients in the diet, and vice versa. In other words, the biological value of a protein or protein mixture will increase as the percentage of protein in the diet decreases.

It may also be presumed that conditions modifying the glandular activities of the body will change the measured biological values of protein and mixtures of proteins by changing the "particulate" amino acid requirements of the body. Thus, variations in the activity of the thyroid or the adrenal bodies would presumably decrease or intensify the requirement for tyrosine. Variations in liver function, particularly in response to changes in the character of the diet, would presumably decrease or intensify the requirement for cystine, in so far as a variable output of taurocholic acid occurs. In similar manner, the body's require-

[3] Thomas, K., *Arch. Physiol.*, 1909, 219.
[4] Martin, C. J., and Robison, R., *Biochem. J.*, 1922, xvi, 407.
[5] Sherman, H. C., "Chemistry of Food and Nutrition," New York, 1926, 3rd ed., p. 252.
[6] Armsby, H. P., "The Nutrition of Farm Animals," New York, 1917, pp. 384-388.

ments for tryptophane, lysine, histidine and other essential amino acids may vary, particularly with the nutritive condition of the animal.

It seems unreasonable, therefore, to expect a constant utilization in anabolism of a given assortment of amino acids under all conditions. The realization of this unwelcome factor in the problem of measuring and expressing the nutritive value of proteins emphasizes the futility of attempts to gauge the value of proteins in nutrition from chemical studies of their amino acid constitution. The chemical characterization of a protein or of a mixture of proteins occurring in a definite food material is a different problem from its biological characterization, and the results obtained in each case may be but loosely related.

METHODS OF INVESTIGATION

Investigations of the biological properties of isolated proteins and definite protein mixtures have been pursued for many years in a number of different ways. It would be presumptuous for any student of the subject to say that some of the methods used are wholly bad and others wholly good; nevertheless, certain requisites of an effective method that would be accepted at the present time may perhaps be deduced from this accumulated experience. Such a preliminary consideration of methods is essential to the proper evaluation of the experimental data at hand, and is justified also by the fact that it has been included so seldom in reviews of the subject, and so little unanimity of opinion in regard to methods appears to exist among those at present engaged in this line of work. One of the authors [2] has elsewhere offered a critical discussion of this subject, but a somewhat different presentation, including a consideration of still other debatable points, may not be out of place in this chapter.

The Preparation of Experimental Rations

The determination of the nutritive value of an isolated protein, or of the protein value in nutrition of a given food or mixture of foods, generally involves a study of the response of experimental animals to definite dietary régimes, so planned that the responses obtained may be definitely (preferably quantitatively) related to the amount and character of the protein consumed. Hence, the experimental rations should be limited in their nutritive value only by the amount and character of their protein components.

This requirement implies two others. In the first place, it means that the experimental ration should be adequate with respect to all other nutrients, including the essential inorganic ions, the essential unidentified

factors designated as vitamins A, B, etc., and the available sources of energy. This requirement in turn must imply an adequate intake of food as well as a proper balance of nutrients in it. The importance of this requirement is paramount if the feeding experiment is to be protracted, particularly if it is to cover the entire period of growth or the entire life span of the experimental animal or a succession of generations. In these protracted experiments, it is a requirement which appears impossible to impose in any deliberate and intelligent fashion, unless the investigator has the temerity to assume that to his certain knowledge *all* of the essential nutritive factors, aside from the amino acids, are supplied by the non-protein components of his rations. Such an assumption implies that all of the needed inorganic ions are contained in his synthetic mineral mixtures and that vitamins A to E (or G) inclusive are the only other unidentified dietary essentials for maintenance, growth, reproduction, lactation and all other animal functions.

The situation is somewhat different in short feeding experiments, extending at most over only a few weeks. In such a case the importance of meeting this requirement may be negligible if the experimental animals are in good nutritive condition, since possible dietary deficiencies in minerals and vitamins may exert no detrimental effect upon the animal as long as the stores of these factors in its own body may be drawn upon. It certainly is not good technic to neglect to balance experimental rations in the best manner possible under present limitations of knowledge, assuming that the animal tissues can themselves supplement all deficiencies in the ration other than protein. However, it seems unnecessary and unjustifiable to discount severely the results of short experiments not conforming to this requirement, or, as McCollum [7] has done, throw them out of court summarily.

In the second place, the requirement that experimental rations should be limited in their nutritive value only by the amount and character of their protein components implies that the amount of protein consumed be always inadequate to support maximum growth or other function under observation; otherwise it is not a limiting factor, and as soon as it ceases to be a limiting factor, amino acids are being consumed in amounts exceeding the demands of the body and are entering, interchangeably with carbohydrates and fats, into the energy exchanges of metabolism. Under these conditions, any measurement of the biological value of protein will underestimate the truth.

[7] McCollum, E. V., and Simmonds, N., "The Newer Knowledge of Nutrition," New York. 1925, 3rd ed., Chap. V.

Protracted versus Short Feeding Experiments

A matter of great importance in the study of the nutritive value of proteins, both from the standpoint of the significance and value of the results obtained and from the standpoint of experimental efficiency, relates to the length of the period of experimental feeding with reference to the longevity of the experimental animal. Largely due to the influence of McCollum and to the extensive experimental investigations of himself and his colleagues, the impression is gaining ground that the physiological effects of any ration can be established only by "observations on the well-being of men or animals restricted during considerable periods of time to a definite type of diet." [7, page 50]

In substantiation of this viewpoint it is recalled "that diets which fall below the standard of quality necessary to maintain life and vigor over a very great fraction of the span of life of which the species is capable of living, may cause no noticeable deterioration in the physical condition, during even 5 per cent of the normal expectation of life. In order to produce noticeable effects by faulty diet during 4 or 5 per cent of the average life, it is necessary that the faults shall be relatively severe. Yet diets which are just good enough to permit a young animal to develop in what appears to be an approximately normal manner may cause early ageing, and instability of the nervous system. We know that while growth is still in progress, or after it has ceased, the adherence for even brief periods of a few days or weeks to a diet which is unsatisfactory in some degree leads to a deviation from the normal histological structure of the osseous, the nervous, or the circulatory tissues, depending upon the nature and extent of the faults in the food. Such abnormal modification of tissue structure would ordinarily escape observation even today, and certainly a few years ago." [7, p. 57]

The work of McCollum and his associates on the protein deficiencies of foods and the relative protein values of foods and mixtures of foods in nutrition has been planned in full recognition of this viewpoint. In the latest reports of this work [8] the method has been greatly extended to cover observations of the growth, the fertility, the success in the rearing of young, the length of life, the preservation of youthful characters and the stability of the nervous system in experimental animals. These observations have sometimes been carried through the second, third, and fourth generations. Observations upon these heterogeneous characters and functions of animal life enable one, according

[8] McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1921, xlvii, 111, 139, 175, 207, 235.

to McCollum, to differentiate between shades of quality in the protein moiety of the diet "to a degree of refinement not equaled by any other method of study."

In assessing the full significance of the frequent experience of investigators in nutrition that rats may thrive for 5 or 6 per cent of their natural span of life upon rations that ultimately prove to be inadequate, it seems wise to consider the reasons for this deferred result. It may be said at the outset that the inadequacy ultimately revealed has generally been found to relate to nutrients that may be stored in the body in considerable amounts relative to current expenditures, such as mineral matter and vitamin A. The writers are not aware of any instance in which this deferred failure was definitely traced to the protein in the ration, or could reasonably be so traced. In all such cases, it seems highly probable that the inadequacy could be detected in a short period of time if it were possible (as it is with protein) to measure the intake of the nutrient in question, its loss from the body, and the resulting balance; with such information at hand from a short period of observation, ultimate nutritive failure could be predicted with certainty if the balance is persistently negative.

In the case of vitamin A and other unidentified dietary factors, it is impossible to obtain the nutritive balance in the absence of chemical methods for its analysis and of knowledge concerning its end products in metabolism. Therefore, extended feeding trials have been resorted to; but the necessity of such protracted periods of feeding in this case, and in similar cases, cannot reasonably be urged in favor of the view that protein studies also must extend over a considerable fraction of the span of life. In fact, even with vitamin A, Sherman and Munsell [9] have shown that, if the rat is deprived of its store of vitamin in a preliminary period, then constant responses of the animal in weight increment may be obtained with different foods, evidently in proportion to their content of vitamin A, during a period of feeding lasting only 8 weeks.

It appears, therefore, that the necessity of protracted feeding periods in testing the nutritive adequacy of rations, or in determining their ultimate nutritive effects, relates only to the unidentified or unsuspected nutritive factors, though these ultimate effects have been interpreted frequently in terms of known nutrients, such as protein. In the case of protein, there is no reason to doubt that the growth obtained in a few weeks of feeding, or the rate of nitrogen retention in even shorter periods under properly controlled conditions, will be representative of

[9] Sherman, H. C., and Munsell, H. E., *J. Amer. Chem. Soc.*, 1925, xlvii, 1639.

the adequacy of the protein supply. Until much more convincing evidence has been presented favoring the necessity of such protracted experiments in the determination of the nutritive value of proteins than is now at hand, such a cumbersome method of research may be considered as not only unnecessary, but as wasteful of time and money, and, indeed, productive of results difficult if not impossible to interpret in the present state of knowledge of nutrition.

It has been pointed out above that the complete interpretation of the results of protracted feeding experiments with reference to the nutritive value of proteins must be based upon the assumption that all of the requirements for nutrients other than protein for maintenance, growth, reproduction and lactation are contained in the experimental rations. This in turn implies that all of the nutritive requirements of animals are known; an implication utterly without justification.

The objection just considered would be removed if each experiment according to this plan were controlled by parallel observations of the effect of a ration identical with that containing the protein under investigation except for the substitution for it of a standard protein or protein mixture known to be of high biological value. If such a substitution leads to the *perfect* performance of all animal functions throughout life, then any imperfections noted with the ration containing the protein whose nutritive effect is being studied could be attributed to the protein itself, *provided it is shown that the ration has been consumed in adequate amounts throughout the period of observation.* Unfortunately this type of feeding experiment is not commonly controlled in this way.

Even when so controlled it may be doubted whether the protracted feeding experiment is an efficient method of research. A characteristic of scientific methods of study is that, wherever possible, all independent factors in the problem at hand are subjected to separate study, for the purpose of determining the peculiar properties of each and its peculiar responses to varying conditions. But in the protracted feeding experiment no such factoring is attempted. No separate study of the protein requirements of maintenance, growth, reproduction, and lactation is attempted, although in all probability these requirements differ both quantitatively and qualitatively. For different foods, therefore, a protein deficiency manifested by defective growth cannot be compared with a protein deficiency manifested by defective fertility, which in turn is in a different category from a protein deficiency revealed by inadequate milk production. Hence, the summation of the results obtained with reference to these different animal functions cannot be

effected in any logical way, and the comparison of different proteins on the basis of *all* the results obtained seems impossible. The heterogeneity of the experimental observations seems to defeat the purpose of the experiment, *i.e.*, the determination of the relative nutritive values of the proteins studied.

With reference to this determination, protracted feeding of experimental animals obscures rather than clarifies the significance of the results obtained. It may be concluded, therefore, that feeding experiments concerned with the measurement of the nutritive value of proteins need to be only of such length that a good determination of the rate of gain representative of the conditions of protein feeding chosen is obtained, or of the rate of milk production or any other animal function. If attention is confined to the nitrogen metabolism characteristic of a particular protein, the length of the experiment would seem to be determined only by the interval of time required for the adjustment of the animal to the conditions of feeding and for the securing of a representative collection of excreta. No doubt has been expressed, even by McCollum, concerning the adequacy of short periods of observation in the determination of the utilization of protein in digestion; why is the question of deferred effects involved to any greater extent in the determination of the utilization of protein in metabolism?

Food Records and the Control of Food Consumption

A vexed question of technic in nutrition investigations consisting of feeding experiments relates to the value of food intake records of experimental animals and to the necessity of a control of the consumption of comparable rations. On the one hand, there are those who consider that the composition of the experimental rations and the response of the experimental animals when consuming them *ad libitum* constitute the sum total of experimental evidence upon which conclusions need be based. On the other hand, there are those who believe that the physiological response observed must be related to the amount of food (or nutrient) consumed as well as to its composition (or character); in a properly balanced dietary in which the character of the protein component is the limiting factor with reference to the physiological response, it is believed by these investigators that the response observed and measured must be related to the amount of protein consumed as well as to its source.

It will perhaps be admitted by all that the latter type of experiment is more exact than the former. It remains to be determined how satisfactory the former may be assumed to be. A consideration of this

question is particularly important in connection with a study of the nutritive value of proteins, first, because the great majority of published investigations on this subject involve feeding experiments in which no attention is paid to food consumption, much less to its control, and, second, because the investigators undertaking such feeding experiments rarely consider the consequences of this neglect if one may judge from the very occasional reference to it and the still less frequent defense of it.

McCollum and Simmonds in their recent book, "The Newer Knowledge of Nutrition" (pp. 100 to 106), have attempted a defense of the neglect of food consumption records in the type of feeding experiment that McCollum has sponsored for years, involved in the "biological analysis" of foods. The defense rests upon a conclusion of Osborne and Mendel, supported by extensive observations of the food consumption of rats subsisting on synthetic diets of variable composition, that "the intake of the individual is determined in large measure by the energy requirement at any given period." * If this is true with *all* rations, then observed differences in the physiological response secured on properly balanced rations containing equal (but not excessive) percentages of proteins from different sources, may be interpreted as expressions of differences in nutritive value of the proteins used. If the intake of food represents solely the satisfaction of the body's demands for energy, then it may be assumed that food will be consumed in proportion to the size and activity of the animal and to the rate of growth permitted by the character of the protein fed. Thus, the rate of growth observed, as well as the amount of food consumed, will depend upon the biological characteristics of the dietary protein.

It is necessary to realize that the experience of Osborne and Mendel upon which the conclusion above cited was based, relates to the feeding of synthetic rations containing highly digestible constituents and possessing no pronounced flavors. That the same conclusion will apply to rations differing distinctly in texture, odor and flavor, due to the inclusion of considerable percentages of natural foods, cannot be inferred. It is also well to point out that Osborne and Mendel conclude that the intake of food, even with synthetic diets, is determined by the energy demands of the rat and the energy value of the food only

* It may be worthy of note that the final quotation from the work of Osborne and Mendel, which is naively cited by McCollum and Simmonds (p. 106) as disposing of all criticism of their work based on the neglect of food intakes, was taken from a footnote appended to a discussion showing clearly that, with rations containing preparations from cereal foods, entirely erroneous conclusions of the relative values of different proteins may result from the absence of records of food intake. Such a citation may constitute a clever rebuttal argument to the repeated criticisms of Osborne and Mendel, but it cannot be considered a serious contribution to the question at issue.

"in large measure." They later observed [10] that the nutritive balance of the diet is also a determining factor, and a large amount of work by many other investigators has revealed a marked tendency for experimental animals to eat sparingly of deficient diets. The factors determining the intake of food are evidently complex and, in the aggregate, little understood. Therefore, under the most favorable conditions the intake of food can only be approximately predicted, and under conditions involving the feeding of rations containing considerable amounts of natural foods, rational prediction is impossible.

From Osborne and Mendel's later work on natural foods, one does not need to search very far to discover instances in which a mere comparison of gains in weight gives a wrong impression of the relative nutritive value of proteins. In an article on the cereal proteins,[11] for example, a comparison is made of the proteins of the barley, rye, oat and wheat kernels by combining them into rations containing definite percentages of protein, contained entirely in the grain studied, and properly supplemented with respect to minerals and vitamin A. Considering the 8 per cent protein rations, the barley ration in 4 weeks induced an average gain of 54 grams in 7 rats, the oat ration an average gain of 27 grams in 9 rats, the rye ration an average gain of 36 grams in 8 rats, and the wheat ration an average gain of 30 grams in 7 rats. One might conclude from these figures, on the principle adhered to by McCollum, that barley protein is distinctly superior to the other sources of protein, among which no clear distinction may be made. Fortunately in this experiment the food intakes have been reported, and, in particular, the consumption of protein has been related to the gains secured. In Table 49, comparisons among the different cereal proteins have been made, on the basis of all these data, by grouping together those rats gaining very nearly at the same rate.

When this is done, it appears that the greater gains on the barley ration were due to a greater consumption of food, since with rats gaining at the same rate on the rye and oat rations, the consumption of food was no greater than that of the barley rats, and the gain per gram of protein consumed was nearly the same. In fact, there is some reason for suspecting a superiority of rye protein over barley protein.

Furthermore, although the average gain on the oat ration was slightly less than that on the wheat ration, a superiority of oat over wheat protein is clearly indicated on the basis of the gain per gram of protein consumed for rats gaining at approximately the same rate.

[10] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxv, 19.
[11] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1920, xli, 275.

TABLE 49. *A Comparison of Cereal Proteins, Taken from Experiments of Osborne and Mendel.*

Source of Protein	Initial Weight gms.	Gain gms.	Food Consumed gms.	Gain per Gram of Protein Consumed gms.
	In periods of 4 weeks			
Barley	66	46	297	1.9
Oat	70	48	306	2.0
Oat	58	45	313	1.8
Barley	58	42	328	1.6
Barley	76	40	312	1.6
Rye	82	44	328	1.7
Rye	67	43	295	1.8
Rye	74	42	291	1.8
Rye	69	37	304	1.5
Rye	82	36	320	1.4
Rye	75	34	304	1.4
Wheat	65	35	346	1.3
Wheat	62	34	332	1.3
Wheat	67	33	323	1.3
Wheat	67	28	274	1.3
Wheat	67	33	323	1.3
Oat	66	30	246	1.5
Wheat	62	23	234	1.2
Wheat	61	20	275	0.9
Oat	66	22	204	1.3
Oat	65	21	204	1.3
Oat	58	20	189	1.3
Oat	69	19	235	1.0
Oat	61	19	184	1.3
	In periods of 10 weeks			
Barley	66	114	917	1.6
Barley	76	112	964	1.5
Oat	70	115	824	1.7
Rye	76	81	711	1.4
Rye	84	77	734	1.3
Rye	74	76	724	1.3
Rye	69	75	728	1.3
Rye	67	73	688	1.3
Wheat	66	84	873	1.2
Wheat	62	83	848	1.2
Wheat	67	78	815	1.2
Wheat	65	77	873	1.1

The results of the 10-week feeding, disregarding unexplainable mortality and refusal of feed in many cases, lead to essentially the same conclusions as those of the shorter period. The superiority of rye protein over wheat protein is clearly demonstrated in both periods on the basis of these closely comparable results.

Many similar instances of the misleading character of the rate of gain of rats on comparable rations may be cited, particularly from the experiments of Hoagland and his associates. All of these experiments emphasize the importance of determining the *amount* of food required to induce the gains observed, and of *using* such information in a quantitative fashion in decisions concerning the relative nutritive value of the rations compared.

In the field of protein nutrition and in many other fields of physiological research, there is at the present time an alarmingly widespread use of feeding experiments on laboratory animals involving no con-

sideration of the effect of the amount of food consumed upon the results secured. This neglect implies that the amount of food an animal consumes has no effect upon growth and hence is not legitimate experimental evidence and may be disregarded in the interpretation of the results observed, or else it implies, in an equally erroneous fashion, that the amount of food an animal consumes is determined solely by its value in nutrition. An experimental animal is considered as a sort of biological reagent of marvelous accuracy and of varied uses, capable of giving as significant a response in body weight change, or in reproductive performance, to a change in ration, as the color response of a chemical indicator to a change in hydrogen-ion concentration around the point of neutrality.

Unfortunately this conception of the infallibility and sensitivity of experimental animals cannot go unchallenged, and methods of experimentation gratuitously involving this conception are not capable of throwing any clear light upon the problems in connection with which they are employed, because they are not properly controlled.

In meeting the criticisms above discussed, the mere recording of food intakes is not sufficient. In fact, it is probable that one reason why more attention is not given to this matter is that the use of such records in the formulation of conclusions is difficult and hence their value is obscure.

In the case of food intake records of individual animals, it may be possible, as was done in Table 49, to select animals for comparison on the basis of comparable gains, or to make the selection on the basis of comparable food intakes. In starting an experiment of this character, however, involving no control of food intakes, the investigator is leaving to chance what should be definitely provided for in the plan of his experiment.

The uncertainty as to the use to which food intake records may be put is entirely removed if the food intakes of comparable animals are controlled, so that the physiological performance of animals on equal amounts of rations differing only in the source and quality of their proteins may be directly determined.

Further discussion of the methods used in experimental studies of the nutritive value of proteins will be found in a recent review by one of the writers.[2] The importance of an understanding of the principles underlying methods of research, of the assumptions involved, and of their debatable features to a fair appraisal of the progress gained, is nowhere better illustrated than in this general field of study.

THE NUTRITIVE VALUE OF PROTEINS FOR MAINTENANCE

The requirements of the animal body for amino acids for growth are such that a shortage of any one amino acid limits the utilization of all the others. Certainly the main function of dietary amino acids in growth is to furnish the units or "building stones" for the construction of protein molecules. The requirement of the animal body for amino acids for the maintenance of the composition of its tissues, however, appears to be in quite a different category, in that, while certain amino acids are indispensable for the complete maintenance of the tissues, their absence from the diet or their presence in inadequate concentration does not prevent the utilization of the other amino acids, though possibly this utilization is to some extent impaired. In this function, apparently, amino acids are serving as precursors of relatively simple nitrogenous constituents of the tissues.[12] Whether the amino acid requirements for maintenance are simpler than those for growth cannot at present be said; it is obvious, however, that some amino acids, such as lysine and cystine, are required in relatively greater amounts for growth than for maintenance.

The relative value of different proteins for maintenance has been investigated rather extensively on rats by McCollum and Simmonds,[13] who have concluded that the cereal proteins are distinctly less efficient for this purpose than the proteins of milk, while the proteins of the flaxseed, navy beans, and peas are distinctly less efficient than those of the cereal grains. It is evident, however, from the charts illustrating the results of this experiment that maintenance of weight was secured in only a small minority of the animals used, and that the distinctions made between different sources of protein are often difficult to detect. In the absence of food records, the results secured are difficult to evaluate.

Using the criterion of continued maintenance of body weight, Osborne and Mendel have reported[14, 15] some studies on the relative values of lactalbumin, casein, edestin, gliadin and the mixed proteins of milk. Expressing the maintenance requirement as the smallest intake of protein per gram of rat per week consistent with constant body weight for six weeks or more when the energy intake was liberal, the average values obtained with uncontrolled food intake were 12 mgms. for lactalbumin, 19 mgms. for casein, 18 mgms. for edestin, 17 mgms. for the mixed proteins of milk, and 24 mgms. for gliadin. In a later

[12] Mitchell, H. H., Nevens, W. B., and Kendall, F. E., *J. Biol. Chem.*, 1922, lii, 417.
[13] McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1917, xxxii, 347.
[14] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1915, xxii, 241.
[15] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1916, xxvi, 1.

experiment, in which the rats received a constant energy intake, the average values found for lactalbumin, casein and edestin, respectively, were 12.5 mgms., 16.4 mgms. and 15.0 mgms., and in another series of trials in which conditions were even more nearly identical, the average values obtained were 9.3 mgms., 15.5 mgms. and 13.8 mgms. Considerable variations were noted among individual rats, so that the significance of the average figures given above may not be great, while the interrelation between energy intake and protein minimum also prevents assigning a definite value to any one protein.* However, it seems clear that the lactalbumin ration was a better source of protein than either the casein or the edestin ration, while of the latter two, the edestin ration seemed slightly superior to the casein ration. The difficulty in interpreting these results in terms of the isolated proteins themselves lies in the fact that all rations used contained considerable amounts of nitrogen, relative to the nitrogen in the protein tested, in largely unknown forms in the "protein-free" milk, constituting about one-fourth of the rations. The extent, if any, of the supplementing effect of the nitrogenous compounds contained in this product prevents any definite interpretation of these otherwise excellent experiments.

This criticism does not apply, however, to the results of similar experiments on the value for maintenance of the proteins in the wheat kernel and its milling products,[16] since no "protein-free" milk was used in compounding the rations. While the individual experiments were admittedly too few and too variable to permit the formulation of very definite conclusions, it seems evident that for the whole wheat kernel about 23 mgms. of protein per gram of rat per week were required for maintenance, for the wheat embryo somewhat less than 20 mgms., and for the proteins of the endosperm (wheat flour) about 23 mgms. or slightly more. It is noteworthy that for maintenance, the proteins of the wheat endosperm are about as efficient on this basis as the proteins of the entire kernel, though when allowance is made for differences in digestibility, the inferiority of the endosperm proteins would be more evident.

There are several metabolism experiments reported in the literature that indicate a practically complete utilization of the nitrogen of several proteins and protein mixtures. McCollum and Steenbock[17] have reported that the nitrogen excretion of the pig on a nitrogen-free diet

* These experiments suggest an interesting relation, hitherto uninvestigated as far as the authors are aware, between the balance of nutrients in a ration and the utilization of its energy. The energy intake of these rats was liberal enough to support considerable growth when the protein concentration was increased.

[16] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 557.

[17] McCollum, E. V., and Steenbock, H., *Wis. Agr. Sta., Res. Bull.* 21, 1912

is not appreciably changed when amounts of corn and oat protein are introduced into the ration (with a withdrawal of isodynamic amounts of starch) equivalent in nitrogen to but little more than the endogenous losses. With wheat proteins a small increase occurred, amounting to 10 or 15 per cent. Steck [18] has likewise shown in experiments on himself that ingestion of small amounts of nitrogen in the form of meat, egg albumin and casein, equivalent to the endogenous loss, had no appreciable effect on the excretion of nitrogen in the urine; in fact, a much larger amount of meat nitrogen still had no such effect. Such results indicate a very complete utilization for maintenance of the nitrogen of these proteins and also of the non-protein nitrogen of meat. However, hemoglobin in equivalent amounts caused marked increases in the urinary nitrogen, indicating a less complete utilization of the nitrogen of this protein. Mitchell [19] has shown, in metabolism experiments on rats, that the ingestion of small amounts of lactalbumin and of the mixed proteins of milk did not cause increases in the urinary nitrogen over that observed in periods of nitrogen-free nourishment.

Other experiments in which the protein intake is considerably larger in proportion to the endogenous losses, have revealed rather distinct differences in nutritive value among different proteins and protein mixtures in covering the maintenance requirements for protein. The most complete experimental investigation of this type that has ever been reported is that of Thomas.[3] The results of Thomas are frequently quoted in textbooks and monographs on nutrition, obviously not because of their inherent accuracy and reliability, but apparently because they are quite comprehensive and have been very conveniently condensed into a tabulated summary of "biological values," representing the percentages of the absorbed nitrogen of each of the foods capable of replacing the nitrogen lost to the body in the course of the endogenous catabolism. The experiments were performed on Thomas himself. To the proteins of milk, meat and fish, Thomas assigns values approximating 100, indicating complete utilization of the absorbed nitrogen. On the other hand, to the proteins of corn and wheat have been given values of 30 and 40, respectively, while the proteins of rice, potatoes and peas are graded, in order, 88, 79 and 56. Thus, according to Thomas, animal proteins are two to three times more valuable in adult nutrition than the cereal proteins.

A study of the details of the experiments upon which these biological values are based, however, detracts greatly from their signifi-

[18] Steck, H., *Biochem. Z.*, 1913, xlix, 195.
[19] Mitchell, H. H., *J. Biol. Chem.*, 1924, lviii, 873.

cance. The periods in which the different foods tested constituted practically the sole source of nitrogen were of one to four days' duration only, with no transition or preliminary periods. Apparently no attempt was made to insure a constant intake of nitrogen and calories during these short periods, nor were the different forms of nitrogen fed at comparable levels, considerable variations in calorie and protein intakes occurring from day to day throughout the experiments. Thus, the vitiating effect of the well-known lag in nitrogen excretion held full sway. The data are taken to indicate that protein nitrogen is better utilized when ingested in small portions several times a day than when ingested in one meal; however, this distributed intake was practiced only with certain of the animal foods, *i.e.*, meat, nutrose, milk, fish and crabs. Hence, the other foods are placed at a disadvantage, regardless of the metabolism data. Furthermore, in computing the average biological values for the proteins of the different foods, what seems to be a purely arbitrary selection of some days and rejection of others was practiced, resulting in an exaggeration of the difference in nutritive value between animal and vegetable proteins. As Hindhede has pointed out,[20] the data are indeed so variable that by selecting certain days it may be shown that meat protein is about twice as valuable as wheat protein, while a selection of other days would lead to just the opposite conclusion. The selection of some experimental results in preference to others is always a hazardous undertaking.

In experiments on rats [21] Mitchell has recently shown that when the protein constitutes but 5 per cent of the ration, permitting maintenance or only very slow growth, the proteins (nitrogen) of milk exhibit a biological value of 93, those of corn a value of 72, those of oats a value of 79, those of rice a value of 86, those of potato a value of 68, those of yeast a value of 85, while casein alone gave a value of only 71. These determinations do not indicate any extreme differences between the biological values of milk proteins and of vegetable proteins in adult nutrition.

Hindhede's experiments [20, 22] on men also indicate a good utilization of vegetable proteins in adult nutrition. On potato as the sole source of nitrogen, equilibrium was attained on a urinary excretion equivalent to 0.048 and 0.064 gram of nitrogen per kgm. of body weight; on rye bread as the sole source of nitrogen, equilibrium was attained on a urinary excretion of 0.047 and 0.050 gram of nitrogen per kgm. These values were obtained on 25 grams or less of digestible protein

[20] Hindhede, M., *Skand. Arch. Physiol.*, 1914, xxxi, 259.
[21] Mitchell, H. H., *J. Biol. Chem.*, 1924, lviii, 905.
[22] Hindhede, M., *Skand. Arch. Physiol.*, 1913, xxx, 97.

per 70 kgm. body weight, assuming all the nitrogen of the feces to be food nitrogen. Essentially the same results on potato nitrogen have been reported by Rose and Cooper [23] in this country, though in their experiment the digestible protein intake was somewhat higher, *i.e.*, 29 grams per 70 kgm. body weight. The urinary nitrogen was equivalent to 0.060 gram per kgm. of weight.* While Abderhalden and associates [24] have obtained results almost identical with those of Rose and Cooper for potato nitrogen, their results with the proteins of rye bread, in contradiction to the data of Hindhede, indicate a distinctly less efficiency. White bread proteins appeared to be no more efficient than rye bread proteins in this regard.

Sherman and his associates [25, 26, 27] have reported the results of metabolism experiments on human subjects demonstrating the attainment of nitrogen equilibrium on 35 to 40 grams of protein per 70 kgm. body weight, consisting to the extent of approximately 90 per cent of the proteins of cornmeal and oatmeal with 10 per cent of milk proteins, or consisting entirely of the proteins of white bread containing an undetermined proportion of milk proteins. According to Sherman, it does not appear to be necessary to discriminate against cereal proteins in the nutrition of adult human beings, since these proteins, with minimal additions of milk protein, seem to function in the metabolism of maintenance with an efficiency comparable with that of the protein of mixed diets.

However, the superiority of animal proteins alone in adult human nutrition is indicated in the work of Dienes,[28] who has shown that in the human subject, after extreme emaciation, more favorable nitrogen balances were obtained on a meat diet than on a diet of wheat or peas. Rose and MacLeod,[29] in experiments on three women subjects, have studied the nitrogen metabolism on diets, adequate in energy, containing practically all of their nitrogen in the form of lean beef, milk, bread and milk, or soybean curd. The nitrogen intake was less per kgm. of weight (0.075 to 0.081 gram) than the average for adult maintenance on a mixed diet determined by Sherman,[25] although in all cases the average nitrogen balance, in periods of 12 to 15 days' duration, was

[23] Rose, M. S., and Cooper, L. F., *J. Biol. Chem.*, 1917, xxx, 201.

* Kon and Klein (*Biochem. J.*, 1928, xxii, 258) have more recently reported an experiment in which two adults, a man and a woman, lived over a period of 167 days in good health on a diet in which the nitrogen was almost entirely derived from potato. Nitrogen equilibrium was attained with the man at a level of urinary nitrogen excretion averaging .051 gram per kgm. of body weight; for the woman a slight negative balance persisted at this level.

[24] Abderhalden, E., Ewald, G., Fodor, A., and Rose, C., *Pflüger's Arch. ges. Physiol.*, 1915, clx, 511.

[25] Sherman, H. C., *J. Biol. Chem.*, 1920, xli, 97.

[26] Sherman, H. C., and Winters, J. C., *J. Biol. Chem.*, 1918, xxxv, 301.

[27] Sherman, H., Winters, J. C., and Phillips, V., *J. Biol. Chem.*, 1919, xxxix, 53.

[28] Dienes, L., *Biochem. Z.*, 1921, cxxiii, 128.

[29] Rose, M. S., and MacLeod, G., *J. Biol. Chem.*, 1925, lxvi, 847.

positive. With two subjects slightly more favorable balances were obtained on milk than on meat diets, while with the third subject no clear distinction was evident. With one subject the bread and milk diet, containing approximately equal amounts of nitrogen from each food, gave almost as favorable a nitrogen balance as the milk diet; in another subject the soybean diet induced a small positive nitrogen balance, as well as a very low level of nitrogen in the urine (60 mgms. per kgm. of body weight).

While the experiments of Hindhede, Rose and Cooper, Abderhalden, Sherman, and Rose and MacLeod testify to the very low requirement of protein in adult human nutrition, and have been interpreted to indicate a very good utilization in metabolism of certain food proteins, the degree of utilization cannot be estimated in the absence of data on the endogenous losses of nitrogen. Indeed, if the endogenous loss in the urine may be taken as about 0.040 gram daily per kgm. of body weight, the utilization secured in several of these experiments could not have been better than 60 to 70 per cent.

Recently Martin and Robison [30] have attempted to measure the value of the proteins of milk as compared with those of whole wheat bread in replacing the nitrogen lost in the course of the endogenous catabolism. They determined for each of the two subjects the extent of the endogenous loss of nitrogen in urine and feces by subsisting for a week or longer on a diet very nearly nitrogen-free. The effect on the nitrogen balance of the ingestion of increasing amounts of nitrogen in the form of the two foods to be tested was then determined, and the biological value calculated at each level of intake according to one of the formulas of Thomas. The minimum endogenous losses in the urine were found to be 38 and 35 mgm. daily per kgm. of body weight. The proteins of whole wheat bread were found to possess an average biological value of 35 and 31 for the two subjects. Nitrogen equilibrium was not attained until the intake was 10 to 12 grams daily, and the absorbed nitrogen 9.5 to 10.5 grams, the energy intake being 60 calories or more per kgm. body weight. This result is quite at variance with that of Sherman [25] *i.e.*, nitrogen equilibrium on an intake of 6.0 grams of bread nitrogen for a man of 80 kgm. and an energy intake of 34 calories per kgm. The results with milk protein were extremely discordant and hardly explainable, since, starting with nitrogen intakes approximating the endogenous losses, increasing amounts of milk nitrogen had no consistent effect on the negative balances secured until an intake of about 11 to 14 grams was reached, when equilibrium was indicated, the energy

[30] Martin, C. J., and Robison, R., *Biochem. J.*, 1922, xvi, 407.

intake being 46 and 47 calories per kgm. On increasing the energy intake with one of the subjects to 57 calories per kgm., nitrogen equilibrium was approximately reached on 6.84 grams of nitrogen. The biological values calculated for one subject were so low that they were not considered in the final conclusions. With the other subject, an average value of 51 was obtained for those periods in which the increased energy intake prevailed. While these experiments were apparently well executed, the results secured are so anomalous that one is at a loss to evaluate them.

Hindhede [31] has criticized the experiment of Martin and Robison with reference to the low biological values obtained for the whole wheat bread, which, indeed, seem much too low. Hindhede believes that the plan of feeding, involving an increase in the dietary N if equilibrium could not be attained in 3 days, is particularly open to criticism. He himself found that if such low intakes of wheat nitrogen are continued for 3 or 4 weeks, the prevailing nitrogen losses will eventually cease and equilibrium can be attained on a diet containing only 3.50 gram of digestible nitrogen. Thus, the minimum level of nitrogen intake at which, in short periods, equilibrium can be attained does not seem to be a reliable measure of the biological value of a protein of definite source, probably because the endogenous output of nitrogen is variable for the same subject at different times.

Finally, attention may be called to somewhat similar experiments reported by Lauter and Jenke.[32] In short periods of 5 to 7 days duration, it was shown that the ingestion of meat nitrogen in amounts approximating the endogenous catabolism increased the urinary nitrogen but little, indicating a very high utilization of the nitrogenous compounds of meat. Experiments on potatoes and wheat flour indicated a less complete utilization of nitrogen, particularly for the wheat flour, but the shortness of the periods of observation and the incomplete description of the experimental rations detract from the significance of the quantitative comparison attempted.

Morgan and Heinz [33] have reported nitrogen metabolism studies on wheat, gluten and almonds, similar in plan to those of Thomas, Steck, and Lauter and Jenke. The results indicate a remarkably complete utilization of almond nitrogen and a less complete utilization of wheat nitrogen. The feeding periods were of such short duration, however, that the computed biological values can hardly be accorded any considerable significance.

[31] Hindhede, M., *Biochem. J.*, 1926, xx, 330.
[32] Lauter, S., and Jenke, M., *Deutsch. Arch. klin. Med.*, 1925, cxlvi, 173.
[33] Morgan, A. F., and Heinz, A. M., *J. Biol. Chem.*, 1919, xxxvii, 215.

In view of the contradiction existing among several of the experiments reviewed above, general statements of the relative nutritive values of food proteins in adult nutrition cannot be made with assurance. At levels of intake so low that equilibrium is not reached, several animal proteins and even cereal proteins appear to be completely utilized in metabolism. For the attainment of nitrogen equilibrium, however, different proteins appear to possess distinctly different efficiencies. This may possibly be due to the fact that a considerable fraction, possibly most, of the nitrogen requirement for maintenance, is related to the replacement of relatively simple nitrogenous constituents of the tissues (see page 467), and may be satisfied, therefore, by a less complete assortment of amino acids than is required for growth, or, in part, even by ammonium salts. The attainment of nitrogen equilibrium, however, very probably means the satisfaction of the requirement for a certain amount of protein synthesis, particularly for the elaboration of the constituents of the digestive juices.[34] While these proteins are in part subsequently digested and absorbed, losses of nitrogen in both feces and urine would seem to be inevitable. Hence, for the complete maintenance of the tissues, the body's demands for nitrogenous substances are in part quite diversified, perhaps even equalling in this respect the requirements for growth.* The superiority of milk proteins in satisfying these requirements seems evident, though the cereal proteins are not greatly inferior to them. There is, quite evidently, a great need for more investigation of the value of different proteins in adult human nutrition.

THE NUTRITIVE VALUE OF PROTEINS FOR GROWTH: EXPERIMENTAL METHODS

The problem of measuring the nutritive values of proteins for growth is complicated by the fact that the protein requirement for growth is superimposed upon that for maintenance. Hence, in simple feeding experiments, the growth secured is not the *total* effect of the dietary protein consumed, since some of the protein is being used to replace endogenous losses of nitrogen. This purely substitutive disposal of the dietary protein is not related to growth and is not directly measurable.

The result of neglecting this difficultly measurable factor in measuring the growth-promoting value of proteins is well illustrated by the experimental data that have been obtained with the quantitative method

[34] Kestner O., *Z. physiol. Chem.*, 1923, cxxx, 208.

* For these reasons, the biological values obtained for incomplete proteins, not capable of supporting nitrogen equilibrium no matter how much is fed, are not comparable with the biological values of complete proteins. Thus, McCollum and Steenbock (*Wisc. Agr. Exp. Sta. Res. Bull.* 21) have obtained values of 80 for zein and 50 to 60 for gelatin. However, these proteins can serve only the substitutive amino acid functions, not the additive ones.

of Osborne, Mendel and Ferry.[35] In this method the gain in weight secured in rats of similar size in a period of feeding on a properly constituted ration is related to the protein actually consumed. The gain per gram of protein consumed is taken as a measure of the growth-promoting value of the ration.

However, if part of the protein consumed is being used in a substitutive rather than an additive way then the gain in weight secured does not represent the total effect of the protein consumed. Furthermore, it may be inferred that the greater the intake of food (and hence of protein) by rats of similar size, the greater the proportion of the protein intake that will be available for growth, and hence the greater the gain per gram of protein consumed. This point has been discussed in some detail elsewhere [2] by one of the writers. In confirmation of the general correctness of this inference attention may be directed in particular to the work of Osborne and Mendel on the relative values of the proteins of the cereal seeds.[36] In the tables of data, a distinct tendency, in each group of comparable results, for high food intakes and large gains to be associated with high ratios of gain to protein intake may be readily seen.

The determination of the nitrogen metabolism of rats and the computation of the biological value of the protein consumed is an attempt to get around this difficulty as well as to obtain a measure of protein addition in terms of nitrogen rather than in gain in body weight. The gain in weight of a rat may contain variable amounts of fat and hence of protein, and therefore is not a definite physiological effect.[37] Furthermore, Forbes and his associates,[38] in feeding and slaughter experiments on swine, have shown that the protein content of the gain may be a specific effect of the character of the protein fed.

In determining the biological value of a protein the analysis of the loss of protein in digestion and metabolism is, of necessity, based upon an analysis of the losses of nitrogen. Hence, these losses must be referred to the total nitrogen content of the food or ration, rather than to its protein content; the interpretation of the results in terms of protein is a purely conventional interpretation, as is the "crude protein" content itself. The scheme possesses, however, considerable biological justification, since the non-protein nitrogenous compounds of foods, consisting largely of amino acids and their derivatives,[39] in all probability

[35] Osborne, T. B., Mendel, L. B., and Ferry, E. L., *J. Biol. Chem.*, 1919, xxxvii, 223.
[36] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1920, xli, 275.
[37] Mitchell, H. H., and Carman, G. G., *Amer. J. Physiol.*, 1926, lxxvi, 398.
[38] Forbes, E. B., Beegle, F. M., Fritz, C. M., Morgan, L. E., and Rhue, S. N., *Ohio Agr. Exp. Sta. Bull.* 283, 1915.
[39] Grindley, H. S., and Eckstein, H. C., *J. Amer. Chem. Soc.*, 1916, xxxviii, 1425; Jodidi, S. L., and Markley, K. S., *ibid.*, 1923, xlv, 2137; Sharp, P. F., *Cereal Chem.*, 1925, ii, 12.

contribute to the value of the food as a source of nitrogenous compounds for the body.

In estimating the total physiological effect of dietary protein, the dietary nitrogen substituted for that lost from the body in the endogenous catabolism as well as that added to the body in growth is considered. In a method proposed by Mitchell [40] for making these estimations, each experimental animal (rat) is standardized in a period of feeding on either a very low nitrogen diet or on a diet containing a small percentage of dried whole egg, the nitrogen of which at this level of intake may be considered as 100 per cent digestible and 100 per cent utilizable in metabolism. The ratio of urinary nitrogen to body weight in these standardizing periods is used in the periods of protein feeding to estimate the body nitrogen appearing in the urine, the so-called "endogenous nitrogen," while the ratio of fecal nitrogen to food intake in the standardizing periods is used in the periods of protein feeding to estimate the body nitrogen appearing in the feces, the so-called "metabolic nitrogen."

While the biological value of a protein thus takes account of the substitutive as well as the additive disposal of dietary nitrogen, its significance is complicated by the fact that in so doing it becomes a composite measure of value, applying to maintenance as well as growth, and probably varying to some extent with the ratio of nitrogen used for these two purposes.

Thus, in the method of determining the biological value of protein by nitrogen balance studies, as well as in the numerical method of measuring the growth-promoting values of proteins by the gain per gram of protein consumed, the results would gain in significance if they could logically be corrected for the protein used for maintenance, so that the final value would apply to growth only. For example, in the former case the biological value of the protein for growth might be thought of as the percentage of absorbed nitrogen consumed in excess of the requirement for maintenance that is retained in growth; in the latter case, the gain in weight might be related to the protein consumed above the maintenance requirement.

Unfortunately, such a correction cannot be made in any known simple and direct fashion, since as the percentage of protein in a maintenance ration is increased to that of a ration permitting growth, the utilization of the protein decreases. This has been shown by nitrogen

[40] Mitchell, H. H., *J. Biol. Chem.*, 1924, lviii, 873; Mitchell and Carman, G. G., *ibid.*, 1924, lx, 613; *ibid.*, 1926, lxviii, 183.

metabolism studies on rats [41] and is indicated clearly by the ratio of gain to protein consumed for the same protein at different levels of intake,[42] when allowance is made for the increasing proportion of protein used for growth as the percentage of protein in the ration increases.[2, p. 444]

This decreasing utilization of nitrogen in anabolism at increasing levels of intake has been interpreted as a result of a decreasing protein-sparing effect of the carbohydrates and fats with the narrowing of the nutritive ratio of the diet. For all practical purposes, it amounts to an increasing maintenance requirement for protein with increasing concentration of protein in the diet. It is evident, therefore, that the amount of a given protein required to maintain an animal on a ration containing 2 or 3 per cent of protein cannot be used in a subsequent growth experiment with a ration containing 8 or 10 per cent of protein in the estimation of the protein consumed in excess of the maintenance requirement. Thus, no simple method appears to exist for resolving composite measures of the nutritive value of proteins for maintenance and growth into their logical components.

Another obstacle in the path of any method of determining a separate biological value of protein for physiological functions, such as growth and lactation, necessarily superimposed upon the function of maintenance, relates to the very evident qualitative differences between the amino acid requirements for maintenance and growth, and very probably between those for maintenance and milk production.

In serving for the maintenance of the nitrogenous integrity of the tissues, any protein may be considered as consisting, under definite conditions, of two fractions, one destined to be utilized completely in anabolism, the other fraction, because of chemical inadequacy, destined to be deaminized and used in the body for other purposes in common with the non-nitrogenous nutrients. However, if a protein is serving simultaneously for maintenance and growth, of the maintenance quota, the latter fraction, unsuited chemically for maintenance, may nevertheless be utilized in part at least by rendering more efficient for growth the remainder of the protein intake. In other words, the selection for maintenance of certain amino acids in certain proportions may leave a residual mixture of amino acids possessing a higher biological value for growth than the original protein, by reason of containing, for example, a greater concentration of lysine or cystine. Under such conditions, the impossibility of assessing accurately the value of a protein for growth

[41] Mitchell, H. H., *J. Biol. Chem.*, 1924, lviii, 905; Mitchell and Villegas, V., *J. Dairy Sci.*, 1923, vi, 222.

[42] Osborne, T. B., Mendel, L. B., and Ferry, E. L., *J. Biol. Chem.*, 1919, xxxvii, 223; Osborne and Mendel, *ibid.*, 1920, xli, 275.

alone from its determined value for maintenance alone and for maintenance and growth simultaneously, is obvious.

It thus seems impossible to obtain specific measurements of the value of different proteins for physiological functions, such as growth, reproduction and lactation, that must of necessity be superimposed upon the function of maintenance. The task of differentiating the fraction of the protein intake concerned with growth only from that concerned with maintenance, so that the amount of growth secured or of protein added to the body can be related to that amount of dietary protein responsible for it, appears to be hopeless. The alternative course, therefore, is either to relate the amount of growth secured to the total protein intake, or by proper metabolism experiments, to relate the amount of nitrogen apparently retained in the body for both maintenance and growth to the amount of dietary nitrogen apparently absorbed from the intestinal tract. The first method ignores the maintenance requirement entirely; the second gives a composite measure for both maintenance and growth.

THE NUTRITIVE VALUE OF PROTEINS FOR GROWTH: EXPERIMENTAL RESULTS

Using their method for the biological analysis of foods in its most comprehensive form to include observations on growth, the maternal functions, the appearance of the characteristics of senility, and the well-being of succeeding generations, McCollum, Simmonds and Parsons [8] have arranged the proteins of a number of animal and vegetable foods in a series of decreasing biological value, as follows: 1, beef kidney; 2, wheat; 3, milk, beef liver; 4, beef muscle, barley, rye; 5, corn, oats; 6, soybeans, navy beans, peas. It will be observed that the proteins of wheat are assigned a higher value than the proteins of milk or of meat in contradiction to earlier conclusions of the same authors,[43, 44] while the proteins of barley and rye are placed on a par with the proteins of meat.* These relations are quite contrary to those indicated by less ambitious methods of research. One naturally raises the question whether the method used by these investigators is equivalent to other methods hitherto considered adequate for the purpose. To the writers there is little doubt but that growth experiments and nitrogen balance studies, conducted under proper control, can give reliable information of the chemical adequacy of proteins in nutrition. To account for failures in the proper performance of the maternal functions and for nutritive

[43] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xx, 415.
[44] McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1919, xxxvii, 155.

* In the same sort of experiments, G. A. Hartwell (*Biochem. J.*, 1927, xxi, 282) has investigated the dietary value of potato protein, finding it to be capable of supporting slow growth in the rat, but not normal reproduction or lactation. It is admitted, however, that the level of protein feeding may have been responsible for the subnormal nutrition.

failures in the second and third generations, by reference solely to the source of protein in the diet, no matter how complete the diet may *seem* to be in other factors, is equivalent to assuming that present knowledge of nutritive requirements is complete. Such an assumption, however, cannot be justified.* Since most of the factors involved in the final judgment of these comparative values of food proteins are not capable of numerical expression, and even the factor of growth is not so expressed, it is an extremely difficult matter, taking the results at their face value, to check the final conclusions, or to criticize them intelligently.

A large number of experiments have been reported concerning the nutritive adequacy for growth of the proteins of different foods and food materials. It has in general been found that most food proteins, if fed at a sufficiently high level, are capable of supporting normal growth in the rat. With certain leguminous seeds, however, namely, lima beans [45] and navy beans,[46] practically no growth can be secured at levels of intake which induce normal growth with most food proteins; other leguminous seeds, the cow pea,[47] the adzuki bean,[48] and the common vetch,[49] also contain obviously inferior proteins, though the proteins of the common field pea [47] and the soybean [50, 51, 52] are exceptionally good for seeds of this class. The proteins of the entire cereal seeds are adequate for normal growth at moderate levels of intake [16, 36, 50] with the possible exception of corn proteins, which do not seem to have been investigated under the most favorable conditions due to the low content of protein in corn. Hart and McCollum's work on the pig [53] demonstrates that corn supplemented with gluten feed, a protein concentrate prepared from corn, and minerals, is capable of supporting normal growth. The proteins of wheat

* In this connection the following quotation by Osborne and Mendel [36] may be cited: "We have not made observations on the total length of life of animals which have grown up upon a diet furnishing proteins from a single seed like barley, or upon their ability to produce and rear young. We believe that there are too many other nutritive factors involved in successful nutrition to enable us, upon the basis of our present knowledge, to charge any failures of nutrition in the second generation to chemical inadequacy of protein solely, although it is quite conceivable that the level of protein metabolism represented by a relatively high or low content of protein in the ration over a long span of life may have a pronounced effect upon the maternal functions. It seems to us, however, that if an animal is able to attain adult size upon a diet which furnishes protein from a single source, the nutritive value of this protein is clearly established. Our experiments with barley have demonstrated the possibility of attaining large size where proteins from this source alone were available—provided the total intake of food and consequently of protein and energy was adequate. In view of such positive results, in contrast with the less successful growth obtained in experiments made by others and conducted perhaps under less favorable conditions, it seems hazardous to venture a strict comparison of the nutritional value of proteins from the records of different investigators without more precise information of a quantitative nature regarding the relations of body gain to protein intake where the energy and other essential factors are strictly comparable."

[45] Finks, A. J., and Johns, C. O., *Amer. J. Physiol.*, 1921, lvi, 205.
[46] Johns, C. O., and Finks, A. J., *J. Biol. Chem.*, 1920, xli, 379.
[47] Finks, A. J., Jones, D. B., and Johns, C. O., *J. Biol. Chem.*, 1922, lii, 403.
[48] Johns, C. O., and Finks, A. J., *Amer. J. Physiol.*, 1921, lvi, 208.
[49] Sure, B., *J. Biol. Chem.*, 1921, xlvi, 443.
[50] Suzuki, U., Okuda, U., Matsuyama, Y., Okimoto, T., Katakura, M., and Yuwata, M., *J. Tokio Chem. Soc.*, 1920, xli, 381.
[51] Daniels, A. L., and Nichols, N. B., *J. Biol. Chem.*, 1917, xxxii, 91.
[52] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxii, 369.
[53] Hart, E. B., and McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 373.

flour seem to be definitely inferior to those of wheat;[16, 54] the evidence concerning the nutritive value of the endosperm proteins of corn is conflicting, Osborne and Mendel securing very little growth with such proteins,[55] while Johns, Finks and Paul have secured normal growth with commercial corn gluten meal fed at a slightly higher level in a ration containing slightly less fat.[56]

Experiments of the type just considered do not call for any extended comment, since they do not permit of any accurate comparisons of one protein or mixture of proteins with another, the rations used not being comparable in energy value and the actual intake of food not being recorded. It is interesting to note, however, occasional instances in which rations unsuitable for the support of growth or even maintenance were converted into rations suitable for growth or continued maintenance by the simple expedient of cooking (soybean, cottonseed, cow pea, navy bean, lima bean), or the removal of toxic (velvet beans) or distasteful (pecan nuts, walnuts and almonds) ingredients. If these expedients had not been attempted, it is evident that a false idea of the inadequacy of the protein concerned would have been formed, simply due to the fact that the animals were not eating enough of the unmodified ration to constitute a fair test of its nutritive adequacy.

This method of the biological evaluation of food proteins is rendered somewhat more efficient by determining the growth of rats on different proteins fed at different levels of intake, no attempt being made, however, to relate the amount of growth secured to the amount of protein or food consumed. By this method McCollum and Davis [43] have shown that, with rations of comparable energy value (5% added fat), 6 per cent of milk proteins was adequate for normal growth, while 6 per cent of the proteins of the whole wheat kernel gave very slow growth, comparable to that secured with only 4 per cent of milk proteins. Drummond [57] investigated the proteins of cod, herring and salmon muscle as compared with beef muscle proteins and casein, varying the proportion of each protein in diets containing 20 per cent of fat and noting the rate of growth secured. No differences were found in the growth-promoting value of the fish and beef muscle proteins, though casein seemed to be of slightly inferior value. Suzuki and his associates [50, 58] have confirmed these results of Drummond, indicating a similarity in the biological value of fish muscle proteins and beef proteins, and the distinct inferiority of

[54] Johns, C. O., and Finks, A. J., *J. Biol. Chem.*, 1920, xlii, 569.
[55] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1914, xviii, 1.
[56] Johns, C. O., Finks, A. J., and Paul, M. S., *J. Biol. Chem.*, 1920, xli, 391.
[57] Drummond, J. C., *J. Physiol.*, 1918, lii, 95.
[58] Suzuki, U., Okuda, U., Okimoto, T., and Nagasawa, T., *J. Tokio Chem. Soc.*, 1919, xl, 385.

casein. Normal growth was attained in these experiments on rations containing 10 per cent of animal proteins, 28 per cent of a modified "protein-free" milk, and 14 per cent of fat. In these experiments the extracted proteins of rice at a 10 per cent level gave excellent growth.

It seems clear that the results of these uncontrolled feeding experiments are significant in all cases in which successful growth and physiological functioning are secured; in such cases, the protein supply is adequate in all respects beyond a doubt. It is equally true, however, that the significance of unsuccessful results is not unequivocal, since no evidence is offered that the intake of food was adequate for the most successful nutrition, or was comparable with that secured on a more successful ration with which the first ration is compared. It is unfortunate that the limitations of such uncontrolled experiments are not more generally appreciated. Merely for the purpose of illustrating the situation, reports of feeding experiments of this type may be cited in which lactalbumin has been declared [59] an incomplete protein, although its amino acid constitution [60] does not support such a verdict; the value of oatmeal proteins in promoting growth, gestation and lactation has been very nicely assessed; [61] fine distinctions between egg white [62] or egg albumin [63] and casein have been made; a sex difference in the response to differences in the quality of dietary proteins has been discovered [64]; and a relation between protein utilization in anabolism and the intake of vitamin B has been announced.[65] To the writers, it does not appear to be a profitable undertaking to discuss the soundness of these conclusions, since all the experimental evidence has not been reported, even though it may have been obtained. Objection to the method is not removed by the mere recording and reporting of food intakes, except in cases where comparable rations are voluntarily consumed in comparable amounts by the experimental animals. The only way to assure this condition, so essential to an unequivocal interpretation of growth data, is to control the food consumption by restricting the intake of the ration that is the more readily eaten.

A further step in the refinement of biological methods for assessing the protein value of foods, relates the amount of growth secured to the intake of protein. By this method Osborne, Mendel and Ferry [35] have demonstrated the superiority of lactalbumin over casein in promoting

[59] Sure, B., *J. Biol. Chem.*, 1920, xliii, 457.
[60] Mendel, L. B., "Nutrition: The Chemistry of Life," New Haven, 1923, p. 115.
[61] Hartwell, G. A., *Biochem. J.*, 1926, xx, 751.
[62] Mitchell, H. S., *Amer. J. Physiol.*, 1925, lxxiv, 359.
[63] Hartwell, G. A., *Biochem. J.*, 1922, xvi, 78.
[64] Hartwell, G. A., *Brit. J. Exper. Biol.*, 1925, ii, 323.
[65] Reader, V., and Drummond, J. C., *Biochem. J.*, 1926, xx, 1256; Hartwell, G. A., *ibid.*, 1926, xx, 1279. *On the contrary, see* Sherman, H. C., and Gloy, O. H. M., *J. Biol. Chem.*, 1927, lxxiv, 117.

growth, when fed with "protein-free" milk, and Osborne and Mendel have demonstrated the superiority of casein over edestin [15] under similar conditions of feeding. This latter finding is noteworthy, since in covering the maintenance requirement edestin has been found superior to casein (see above). In a later investigation on the nutritive value of the wheat kernel and its milling products,[16] the same investigators obtained about 1.3 grams of gain per gram of protein consumed for the whole wheat kernel at a protein level of 10.3 per cent, a value of 0.5 gram for endosperm proteins at a level of 14.7 per cent, a value of 1.6 grams for wheat embryo proteins at levels of 13.7 and 6.95 per cent, and for one rat a value of 2.05 grams for the proteins of wheat bran at a level of 9.05 per cent. If these values may be taken as entirely comparable—though they cannot be considered as maximum values since the effect of changing the level of protein intake was not sufficiently investigated—they indicate that embryo proteins and bran proteins are superior to those of the whole kernel, while endosperm proteins are markedly inferior. Since the endosperm contains almost three-fourths of the protein of the entire seed, it seems obvious that the bran and embryo proteins must supplement very efficiently the proteins of the endosperm. Murphy and Jones,[66] using the same method of study, have confirmed the high nutritive value of the proteins of wheat bran, particularly as compared with the proteins of patent white flours.

Their investigation of cereal proteins was later extended by Osborne and Mendel [36] to barley, oats and rye, as compared with wheat, using the whole kernel in each case. The maximum average gains per gram of protein secured with the different cereals, with the corresponding levels of intake, were: for barley, 1.89 at an 8 per cent level; for oats, 1.82 at a 5 per cent level; for rye, 1.49 at an 8 per cent level; and for wheat, 1.40 at a 10 per cent level. All of these values were obtained in periods of 4 weeks' duration. The differences indicated by these figures are not considered sufficiently significant by the investigators themselves to warrant any conclusion regarding the relative efficiency of the different cereal proteins. It is pointed out, however, that these values are not greatly less than those secured under comparable conditions for casein and lactalbumin. It has been shown above (Table 49), however, that comparisons of selected data, based upon equal gains in weight, indicate no superiority of barley proteins over rye or oat proteins, but a rather distinct though slight superiority of oat proteins over wheat proteins, all comparisons being made on rations containing 8 per cent of crude protein.

In comparing the values obtained for different proteins by this

[66] Murphy, J. C., and Jones, D. B., *J. Biol. Chem.*, 1926, lxix, 85.

numerical method, it should be remembered that they are affected by differences in the inherent digestibility of the proteins themselves and also by the presence of indigestible carbohydrates in the ration in so far as these substances depress the digestibility of the proteins.

Using the same numerical method of expressing the efficiency of proteins for growth, Morgan, Newbecker and Bridge [67] have made an interetsing tusdy of the proteins of the almond. While the mixed proteins of the almond were adequate for normal growth at a level of 17.2 per cent in rations containing 20 per cent of added fat, the globulin obtained by extracting the ether-extracted almond meal with 10 per cent sodium chloride, was distinctly less efficient; on the other hand, the fat-free residue after sodium chloride extraction and subsequent leaching with water to remove the excess of salt, contained proteins of exceptional efficiency. When fed at a level of 4.5 per cent in a ration the energy value of which is difficult to assess because of the indigestible character of much of the carbohydrate matter in the nut residue, these proteins produced almost normal growth with a ratio of gain to intake markedly higher than that secured with the almond meal. For the rats fed on this ration, an average gain of 3.87 grams per gram of protein consumed was secured in a 4-week period. This exceeds any value thus far reported by this method of expressing protein efficiency. It is interesting to note that, in a comparable investigation of the proteins of the English walnut, Mignon [68] has not discovered any clear distinction in growth-promoting value among the fractional proteins of this nut.

Hoagland and Snider have recently applied the numerical method of measuring the growth-promoting value of proteins of Osborne, Mendel and Ferry in extensive studies of animal foods, particularly animal tissues. In one of their first articles [69] they noted that, throughout a large number of comparisons, the gain in weight per gram of protein consumed was, with only two exceptions, larger for the male rats than for the female rats. This sex difference was correctly interpreted as the result of the greater gaining capacity of male rats as compared with female rats and the resulting greater consumption of food. Hence with the males a larger proportion of the protein consumed was used for growth and a smaller proportion for maintenance than with the females. In their future work, therefore, they used male rats only. However, one important implication from this interesting experimental finding is that, in comparisons of different sources of protein, a great disparity in the food consumption of rats on different rations will, for the same reason,

[67] Morgan, A. F., Newbecker, B. M., and Bridge, E., *Amer. J. Physiol.*, 1923, lxvii, 173.
[68] Mignon, H. L., *Amer. J. Physiol.*, 1923, lxvi, 1.
[69] Hoagland, R., and Snider, G. G., *J. Agr. Res.*, 1926, xxxii, 1025.

obscure the interpretation of the difference observed in the ratio of gain to protein consumed. In other words, this ratio may be markedly affected by the amount of food consumed as well as by the quality of the protein.

The investigations of Hoagland and Snider have established the high biological value of the protein (nitrogen) in the muscle of the calf, the ox, the sheep, and the hog [69, 70] and of the protein in the spleen, heart, liver and kidney. The proteins of these animal products and of whole milk are of equal value for maintenance and growth in so far as this method of measurement was able to discriminate. Less favorable results were obtained with the thymus gland (sweetbreads), tripe, casein, and various miscellaneous animal tissues used mainly in the manufacture of sausage, but the much smaller food consumption on the rations containing these products makes comparison with the other rations difficult. The nitrogenous compounds of beef extract were shown to be inadequate for maintenance,[71] and tissues consisting largely of connective tissue, such as "cracklings," palates, and hog snouts, appeared to contain proteins of inferior biological value. Blood serum proteins gave somewhat more favorable results, but no measure of the nutritive value of the proteins of dried blood and of hemoglobin could be secured, because the rations containing these products were so distasteful to the experimental rats. The extensive experience of Hoagland and Snider illustrates the relative ease in measuring the biological value of proteins when high, as well as the difficulty in obtaining comparable values for inferior proteins.

In their latest contribution,[72] Hoagland and Snider have demonstrated a close similarity in the nutritive value of the proteins of whole wheat and of oatmeal, and the distinct inferiority of the proteins of navy beans (cooked). A low value was also obtained for patent white flour, but the smaller average food consumption of the rats on this ration prevents a satisfactory comparison with the other foods.*

Passing next to investigations of the nutritive value of proteins for maintenance and growth involving a study of the nitrogen metabolism of the experimental animals, the work of McCollum,[73] comparing the proteins of the cereal grains with those of milk in promoting nitrogen retention in the pig, is worthy of special comment. McCollum found that, of the total nitrogen intake, about one-fourth was retained for growth in the case of corn, wheat and oats; of the absorbed nitrogen from the same

[70] Hoagland, R., and Snider, G. G., *J. Agr. Res.*, 1926, xxxii, 679.
[71] Hoagland, R., and Snider, G. G., *J. Agr. Res.*, 1926, xxxiii 829.
[72] Hoagland, R., and Snider, G. G., *J. Agr. Res.*, 1927, xxxiv, 297.
* Kon (*Biochem. J.*, 1928, xxii, 261) has recently applied the numerical method of measuring the growth-promoting value of proteins to "tuberin," the globulin of the potato. The protein was fed to rats at levels of approximately 8, 10, and 12 per cent, and a maximal gain in weight of 2 grams per gram of protein consumed was obtained at a level of 7.8 per cent of protein in the diet.
[73] McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 323.

food materials (in the calculation of which an estimate of the "metabolic nitrogen" of the feces was used) approximately 30 per cent was retained. The data of the individual experiments do not offer any clear evidence of differences in the utilization of the nitrogen of the three cereal grains tested. In one experiment on casein, 46 per cent of the nitrogen intake, equivalent to 51 per cent of the absorbed nitrogen, was represented in the nitrogen balance. In one experiment on skim milk, these percentage were 63 and 66, respectively.

However, when the biological efficiency of a protein is computed in such a way as to neglect the nitrogen used for maintenance, a false impression is conveyed of the differences existing in this respect among different proteins. From such computations the conclusion might be drawn that casein and the mixed proteins of milk are two to three times as efficient as the proteins of the cereal grains. Since the creatinine nitrogen excreted in the urine was determined in these experiments, it is possible to compute the total endogenous nitrogen on the assumption that this is 5.5 times the creatinine nitrogen,[17] and to calculate biological values involving all of the purposes for which nitrogen was used.[19] The results of such a calculation give average values for corn, wheat and oat proteins of 48, 44 and 42, respectively, the value for casein being 67, and for milk proteins 80. These figures are percentages of the absorbed nitrogen presumably used for maintenance as well as growth; while the marked superiority in nutrition of the mixed proteins of milk is still evident, they cannot be said to be as much as twice as valuable as the cereal proteins. Casein itself does not seem to be greatly superior to the proteins of corn, wheat and oats. In fact, in two earlier experiments on pigs reported by McCollum and Steenbock,[17] the percentage efficiency of casein computed in a similar manner is only 57 and 58.

Mitchell [21] has recently reported a series of nitrogen balance studies on rats designed to determine the biological value for maintenance and growth of different food proteins when fed at an 8 to 10 per cent level in rations containing 10 per cent of added fat. The percentage values obtained are entirely comparable with those computed from the metabolism work of McCollum.* For the mixed proteins (total nitrogen) of milk an average efficiency of 85 was found, for corn proteins 60, for oat

* In this work the biological value of the protein represents the percentage of the absorbed nitrogen that escapes excretion in the urine. The difficulty in determining this percentage rests, first, in determining the actual absorbed nitrogen, due allowance being made for nitrogen of body origin excreted in the feces, and second, in determining the urinary nitrogen of body origin. Unfortunately there seems to be no direct method available for differentiating body from dietary nitrogen in either feces or urine, so that indirect methods, involving debatable assumptions, must be called upon. These corrections for body nitrogen may be made [19] on the basis of the nitrogen excretion in feces and urine of animals maintained on a nitrogen-free diet, consumed in amounts to cover the energy requirements for maintenance.

proteins 65, for potato proteins 67,* and for navy bean proteins 38. In earlier papers from the Division of Animal Nutrition of the University of Illinois,[74, 75] values have been reported for the proteins of rice bran 68, cottonseed 66, soybean 64, alfalfa hay 62, and coconut 58. In later experiments [76, 77] Mitchell and Carman have found average biological values at an 8 per cent level of intake of 93 for the proteins of the hen's egg, 83 for the proteins of egg white, 85 for the proteins of milk, 67 for the proteins of wheat, 52 for the proteins of patent white flour, and 74 for the proteins of pork muscle. Under comparable experimental conditions, Mitchell and Beadles [78] obtained average biological values of 75 for beef heart, and 77 for both beef liver and beef kidney. When the level of protein (N x 6.25) intake is increased from 8 to 16 per cent of the ration, the measured biological value of beef liver decreased from 77 to 58, in accordance with previous experience in this direction.[21]

In a continuation of this line of work, the biological efficiency of the protein (nitrogen) in the cacao bean, as it is found in commercial cocoa preparations, was measured by Mitchell, Beadles and Keith.[79] At an 8 per cent level of intake, the nitrogen of cocoa was found to possess an average digestibility of only 38 per cent, after making allowance for the metabolic nitrogen in the feces. The average biological value of the absorbed nitrogen of cocoa was found to be 37, and although this determination was somewhat unsatisfactory because of low food consumption and variable individual results it was confirmed in an indirect fashion on the basis of concordant results obtained on a milk-cocoa ration and calculations involving the assumption of no supplementary relations between the nitrogenous compounds of milk and of cocoa.

The biological value of the protein of meat presents a particularly interesting problem, in view of the variable proportion existing, in different kinds and cuts of meat, between the true muscle tissue, containing proteins of high biological value, and the connective tissue, containing proteins of low biological value. The relation of the connective tissue content of meat to its toughness has been established by the investigations of Lehmann,[80] which showed that the toughness of raw meat depended largely upon its content of collagen and elastin fibers. In so far as the toughness was due to collagen fibers, it could be prac-

* Kon (*Biochem. J.*, 1928, xxii, 261) has obtained an average biological value of 71 for "tuberin," the globulin of the potato, at an 8 per cent level of intake, using the same method of calculation.

[74] Nevens, W. B., *J. Dairy Sci.*, 1921, iv, 552.
[75] Mitchell, H. H., and Villegas, V., *J. Dairy Sci.*, 1923, vi, 222.
[76] Mitchell, H. H., and Carman, G. G., *J. Biol. Chem.*, 1924, lx, 613.
[77] Mitchell, H. H., and Carman, G. G., *J. Biol. Chem.*, 1926, lxviii, 183.
[78] Mitchell, H. H., and Beadles, J. R., *J. Biol. Chem.*, 1927, lxxi, 429.
[79] Mitchell, H. H., Beadles, J. R., and Keith, M. H., *J. Biol. Chem.*, 1926, lxxi, 15.
[80] Lehman, K. B., *Arch. Hyg.*, 1907, lxiii, 134.

tically removed by cooking, resulting in the conversion of collagen to gelatin, although the rate of this conversion is evidently slow at ordinary cooking temperatures.[81] It appeared probable that the proportion of collagen and elastin in meats would also be related to their protein values in nutrition, as indicated, for example, by the biological values of their digestible nitrogen, since it is known that collagen, at least, is an incomplete protein. In so far as this relation exists, it would not be affected by cooking, since the conversion of collagen to gelatin would not presumably affect its nutritive value.

Before the investigation of Mitchell, Beadles and Kruger[82] was undertaken, there was at hand evidence of a circumstantial character that the more fibrous a cut of meat the lower the biological value of its nitrogen would be. Thus, it had been found[77] that a cut of veal, evidently very fibrous when dried, ground and sieved, gave a biological value of only 62, considerably lower than the values obtained with other meat samples, *i.e.,* 69 for a sample of beef and 74 for a sample of pork. Similarly, in unpublished experiments, the biological value of the total nitrogen of a particularly tough and fibrous piece of beef, the lower round (heel) cut from a bull, was found to be only 56, not much higher than the value for white flour, *i.e.,* 52. The most plausible explanation of these variable values was the one suggested by the appearance of the different samples; namely, that an increased connective tissue content decreases the value of the nitrogen in the nutrition of maintenance and growth. The weakness of the argument lay in the absence of *quantitative* data relating to the connective tissue content of the various samples of meat used. With the working out of a method for the determination of the collagen and elastin content of meat[83] sufficiently accurate for the purpose, the opportunity of putting the question to a direct test was, for the first time, at hand. The plan of the experiment here reported was to determine the biological value of the nitrogen of (*a*) a cut of meat of low connective-tissue content, (*b*) a sample of connective tissue itself, and (*c*) a definite mixture of the two, such as would be found in the less desirable cuts of meat.

The biological value of the nitrogen of pork tenderloin, containing a minimal amount of connective tissue, was found to be 79. That of pork "cracklings," consisting largely of connective tissue, was found to be 25. When the two materials were mixed in the proportion of 3 parts of

[81] Bogue, R. H., "Conditions affecting the hydrolysis of collagen to gelatin." Colloid Symposium Monograph, Madison, Wisc., 1923. p. 265.
[82] Mitchell, H. H., Beadles, J. R., and Kruger, J. H., *J. Biol. Chem.,* 1927, lxxiii, 767.
[83] Mitchell, H. H., Zimmerman, R. L., and Hamilton, T. S., *J. Biol. Chem.,* 1926-27, lxxi, 379.

tenderloin nitrogen to 1 part of "cracklings" nitrogen, a distinct depression of the biological value of the tenderloin nitrogen was observed, the mixture possessing a value of 72.

Thus, cuts of beef varying widely in their content of connective tissue will also vary in the biological values of their nitrogen, so that the less desirable tougher cuts, containing large amounts of connective tissue, would be distinctly less valuable as sources of protein for maintenance and growth. This is not so true of pork, since different cuts of pork do not seem to vary greatly in their content of connective tissue.[82]

A study of the biological value of the proteins of milling products from wheat and corn, consisting of nitrogen metabolism experiments on rats, has been but lately reported by Klein, Harrow, Pine and Funk.[84] Average values obtained with 8 rats were 57 for wheat bran, 61 for standard middlings, 77 for red dog flour, 67 for "second clear," 49 for "first clear," and 47 for patent flour. The consistently high values secured for red dog flour, consisting largely of the aleurone layer of the kernel, higher than any values heretofore reported for vegetable foods of any kind, are notable. The relatively low values for wheat bran are also somewhat surprising. Corn meal, presumably degerminated, showed an average value of 56 in the case of a group of four rats; for the second group of four rats, very variable results were secured, not susceptible of satisfactory averaging. The proteins of corn feed meal, a product the nature of which is not defined though the term possesses no definite meaning, were found to have an average biological value of 60. These values were all obtained with rations containing approximately 8 per cent of crude protein.

Attention has been called to the vitiating effect of variable food consumption on the significance of the ratio of gain in weight of experimental animals to protein consumed as a criterion of the quality of the protein tested. The question naturally arises as to whether the same is true with reference to the biological value of proteins as determined from nitrogen balance studies. Mitchell [21, p. 916] has called attention to the fact that a variable food intake for rats of approximately the same size appears to exert no effect on the biological values obtained, provided the percentage of protein in the experimental ration is the same. However, if the amount of food consumed is inadequate for the maintenance of weight, unreliable biological values may be obtained.

It is also significant [19, p. 893] that rats differing greatly in size give approximately the same biological values for the same experimental rations. Therefore, the biological value of dietary nitrogen seems to be

[84] Klein, A., Harrow, B., Pine, L., and Funk, C., *Amer. J. Physiol.*, 1926, lxxvi, 237.

less subject to disturbance by variations in experimental conditions than the ratio of gain in weight to the amount of protein consumed.

DO THE BIOLOGICAL VALUES OF PROTEINS FOR DIFFERENT ANIMALS DIFFER GREATLY?

Any consideration of the general significance of the biological values of proteins here discussed must include the question whether different species of animals exhibit different efficiencies in their utilization of protein in anabolism. There are some data on rats and pigs for a few foods and combinations of foods that permit a comparison of these two species. The biological values that have obtained with rats for the proteins of corn at a 10 per cent level of intake average about 58, with individual variations ranging from 46 to 69. From Wisconsin data an average value of 48 for five pigs has already been cited. From another publication by Hart and Steenbock [85] designed to test the supplementary relations between the proteins of corn and those of milk, it is possible to compute * the biological values of the different mixtures of proteins, using average figures previously obtained at the same laboratory for the endogenous nitrogen of pigs. These computations are included in Table 50. It will be seen that for the mixture of corn proteins 96.8 and milk

TABLE 50. *The Biological Efficiency of Corn and Milk Fed in Different Proportions to Swine.*

(From published data of E. B. Hart and H. Steenbock.)

Percentage of Corn and Milk Nitrogen in Ration		Per Cent Protein in Ration	Nitrogen Balance in Per Cent of		Per Cent of Absorbed N Used for Maintenance and Growth[a]
Corn	Milk		Nitrogen Intake	Absorbed Nitrogen	
96.8	3.2	8.6	21	40	64
93.6	6.4	8.9	22	40	66
88.3	11.7	9.4	25	46	72
79.3	20.7	10.2	37	56	73
71.5	28.5	10.9	60	73	87

[a] These values have been calculated by the authors from average losses of endogenous nitrogen by pigs reported previously by Wisconsin investigators.*

proteins 3.2 the biological value is 64, while for the mixture of corn proteins 93.6 and milk proteins 6.4 the value is 66; it, therefore, seems fair to assume that, in this experiment with rations containing about 9 per cent of protein, the efficiency of the corn proteins alone was about 60. Mitchell and Kick [85a] * have obtained an average biological value of

[85] Hart, E. B., and Steenbock, H., *J. Biol. Chem.*, 1920, xlii, 167.

* In all of the calculations of the biological values of protein from the Wisconsin metabolism data on swine, we have assumed the following factors for the endogenous nitrogen losses in the urine for pigs of different weight: 40 to 65 lbs., 2.30 grams per 100 lbs.; 65 to 75 lbs., 2.25 grams; 75 to 85 lbs., 2.20 grams; 85 to 95 lbs., 2.10 grams; and 95 to 105 lbs., 2.00 grams. These factors have been selected from various Wisconsin publications on the nitrogen excretion of pigs maintained on a ration of starch and salts.

[85a] * Mitchell, H. H., and Kick, C. H., *J. Agr. Revs.*, 1927, xxxv, 857.

54 for the proteins of corn in experiments on 8 pigs consuming rations containing approximately 8 per cent of crude protein. It may perhaps be concluded, therefore, that rats and swine utilize the absorbable proteins of corn in anabolism with about the same efficiency.

We have some analogous experiments on chickens relating to corn proteins. These data are summarized and analyzed in Table 51.

TABLE 51. *The Biological Value of the Proteins of Corn for Chickens.*

Hen	Average Weight	Daily Food Intake	Daily Nitrogen Intake	Daily Nitrogen Excretion	Nitrogen Excretion on N-Free Ration	Biological Value of Protein
	gms.	gms.	mgms.	mgms.	mgms.	per cent
129	2126	44	717	613	298	56
129	2269	50	815	715	295	56
130	1610	44	717	624	338	60
132	1407	42	685	545	295	62
135	1732	44	717	606	260	52
74	2144	50	815	715	300	49
85	2243	75	1223	992	292	43
151	2492	75	1223	862	249	50
					Average	54

The metabolism data were all obtained in collection periods of 7 days preceded by several weeks of corn feeding. The ration was coarsely ground yellow corn either alone or supplemented by 2 grams of a mineral mixture per bird daily, and contained approximately 10 per cent of crude protein. Since no attempt was made to separate the urinary from the intestinal excretions the calculation of biological values was necessarily different from that used for rats and swine, and may be best explained by illustration. For hen 129, the food nitrogen excreted was taken as $613 - 298 = 315$ mgms., and that used in the body for anabolic purposes as $717 - 315 = 402$ mgms. Assuming the food nitrogen to be totally digestible, the biological value, therefore, is $(402 \div 717) \times 100 = 56$. That this assumption is not greatly in error appears probable from the fact that the true digestibility of corn proteins by the rat seems to be about 95 per cent.[19] Assuming this degree of digestibility in the hen, raises the biological value about 3 points, so that the average for all hens, instead of being 54, would become 57. Ackerson and Blish [86] have reported an average biological value of 68 for the proteins of corn in metabolism studies on 43 mature hens. However, the level of protein feeding must have been much lower than 10 per cent, although the ration given is not described in sufficient detail to permit an exact statement on this point. Possibly the average value of 68 may be compared

[86] Ackerson, C. W., and Blish, M. J., *Poultry Sci.*, 1926, v, 226.

with the value of 72 obtained by Mitchell for rats on a 5 per cent protein ration. It thus appears probable that the hen utilizes the nitrogen of corn in anabolism with about the same efficiency as the rat and the pig.

In this connection it should be mentioned that the biological values reported by Mitchell and associates for oat and wheat proteins for rats are considerably higher than those computed from McCollum's metabolism data on swine. However, the value for milk proteins at a 10 per cent level, 85, checks reasonably well with the computation from McCollum's experiment in which the milk proteins constituted 15.5 per cent of the ration, the biological value in this case being 80. The comparison between the two species can be extended a little further.[87] Nevens [74] has reported a biological value of 58 for the rat for a mixture of corn and alfalfa proteins in equal proportions; Hart and Steenbock [88] have published metabolism data on swine, from which it may be computed, by the use of average factors for the endogenous nitrogen losses of the pig, that a mixture of corn and alfalfa proteins in the proportion of 64 to 36 has a biological value of 63. Similarly, a mixture of corn and milk proteins in the proportion of 3 to 1 has a biological value of 76 for the rat [89] and a value of 80 for the pig. Corn and tankage proteins in equal proportions have a biological value of 65 for the rat, and, in approximately equal proportions, a value of 68 for the pig.

The preponderance of available evidence seems to favor the view that different species of animals utilize proteins in anabolism with about equal efficiency, with the possible exception of ruminants. In these animals the microörganisms of the paunch may multiply to such an extent, at the expense mainly of *non-protein* nitrogenous material, as to modify distinctly the biological value of the nitrogenous material that is ultimately digested and absorbed.

THE RELATION OF THE AMINO-ACID CONSTITUTION TO THE BIOLOGICAL VALUES OF PROTEINS

It is a perfectly obvious proposition that the biological value of a protein is primarily limited by the proportions in which the amino acids that cannot be synthesized by an animal occur in its molecule. This chemical limitation is such that for each protein or mixture of proteins a deficiency in one of the indispensable amino acids curtails its value for structural purposes in the animal body. When this deficiency is remedied by the addition of the appropriate amino acid, a deficiency of some other amino acid may constitute a second limiting factor, and so forth.

[87] Mitchell, H. H., *Proc. Amer. Soc. Animal Production*, 1922, 55.
[88] Hart, E. B., and Steenbock, H. *J. Biol. Chem.*, 1919, xxxviii, 267.
[89] Mitchell, H. H., *J. Biol. Chem.*, 1924, lviii, 923.

Considerable success has, in some instances, rewarded attempts to relate the biological inferiority of certain proteins with definite amino-acid deficiencies. The deficiency of zein in tryptophane and lysine,[90] of gliadin in lysine,[91] of casein in cystine,[92, 15] and of edestin in lysine,[92] has been clearly indicated by feeding experiments with rats reported by Osborne and Mendel. Sherman and Merrill [93] have reported uncontrolled rat feeding experiments indicating that the mixed proteins of milk are also deficient in cystine. Hogan [94] has shown that the first limiting chemical factor in the utilization in metabolism of the mixed proteins of corn is tryptophane and the second limiting factor is lysine. While this experiment would be more conclusive if the intake of protein had been recorded and controlled, the responses to amino acid additions and withdrawals seem sufficiently prompt and distinct to warrant the interpretation given.

A satisfactory demonstration that the mixed proteins of beans of the genus *Phaseolus* are deficient in cystine and that the addition of this amino acid has a marked effect in improving their biological values, has been furnished by Johns and Finks for the navy bean,[46] and particularly for its main globulin, phaseolin, for the adzuki bean [48] and for the lima bean.[45] Distinct indications of a deficiency of cystine in the proteins of the cow pea *Vigna sinensis,* but not of the field pea, *Pisum sativum,* were also reported,[95] and later [96] the same amino acid deficiency was shown for the lentil, *Lens esculenta Moench,* by Jones and Murphy.

For such demonstrations, records of food intake are almost essential. If the difference in the growth secured with the unsupplemented and the supplemented proteins is constant and marked, the obvious conclusion has been established with a high degree of probability, but the final proof must rest in the demonstration that the gain per gram of protein consumed is greatly increased, out of all proportion to the increased food intake, or better yet, that on equal food intakes the addition of the supplementing amino acids has a pronounced effect in increasing the gains secured. The supplementing effect of amino acids may also be clearly demonstrated by suitable metabolism studies.[97, 98, 21]

Many experiments may be found in the literature purporting to show that certain proteins are deficient in certain amino acids, when, as a matter of fact, the results defy intelligent interpretation, because food

[90] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.,* 1914, xvii, 325.
[91] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.,* 1912, xii, 473.
[92] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.,* 1915, xx, 351.
[93] Sherman, H. C., and Merrill, A. T., *J. Biol. Chem.,* 1925, lxiii, 331.
[94] Hogan, A. G., *J. Biol. Chem.,* 1917, xxix, 485.
[95] Finks, A. J., Jones, D. B., and Johns, C. O., *J. Biol. Chem.,* 1922, lii, 403.
[96] Jones, D. B., and Murphy, J. C., *J. Biol. Chem.,* 1924, lix, 243.
[97] Lewis, H. B., *J. Biol. Chem.,* 1917, xxxi, 363.
[98] Lewis, H. B., *J. Biol. Chem.,* 1920, xlii, 289.

intake records are not reported, and the differences in the rate of growth on unsupplemented and supplemented rations are neither invariably secured nor particularly marked when they do appear. This criticism applies with particular force to experiments in which the unsupplemented protein is fed for a time, and then a change is made to another ration in which small amino acid additions are incorporated. A slight response in growth may result in such a case simply because the offering of a freshly made ration may induce a greater intake of food; or possibly certain labile constituents of the first ration may have undergone deterioration in the course of time with a consequent slowing of growth in the experimental animals, and a prompt response in growth when a fresh ration adequate in these unknown constituents is available.* When responses to amino acid additions in experiments of this type are not secured in all cases, the plea that this may have been due to irreparable injury caused by previous amino acid deficiencies, or to individual inability properly to "conserve" the added amino acid, adds nothing to the argument. The mere fact that so many possible explanations may account for the results secured is sufficient proof that experimental conditions were not sufficiently described nor controlled.

Such investigations of the amino acid deficiencies of proteins have yielded results that are difficult to understand and to reconcile with the accepted facts of nutrition. Sure [99] believes he has demonstrated that cystine is the first limiting amino acid in the physiological utilization of lactalbumin, although this protein contains 4.08 per cent of cystine, an exceptionally high content.[100] The same investigator reports a deficiency of proline in edestin,[101] a protein containing at least 4.1 per cent of proline,[60] and no such deficiency in arachin, a globulin from the peanut, although only 1.37 per cent of proline has been isolated from this protein.[102] Sure's experimental results have led him to the belief that amino acid deficiencies not demonstrable at certain high but not excessive levels of intake may be revealed at lower levels by proper amino acid additions.[101]

In much of his work, Sure has introduced a modified method for studying the amino acid deficiencies of proteins, consisting of adding to the food or protein the incomplete protein, gelatin, to the extent of

* See Steenbock, Hart, Sell and Jones, *J. Biol. Chem.*, 1923, lvi, 385. The following quotation is of interest in this connection: "We desire to call attention to the irregularity of growth on the basal ration which we were at a loss to understand until we looked up our data on consumption. We then found that each time that the animals gained weight the ration had been made up fresh. Apparently the cod liver oil of the ration had undergone deterioration in vitamin content with aging. Of the deterioration there can be no question. . . ."

[99] Sure, B., *J. Biol. Chem.*, 1920, xliii, 457.

[100] Jones, D. B., Gersdorff, C. E. F., and Moeller, O., *J. Biol. Chem.*, 1924, lxii, 183.

[101] Sure, B., *J. Biol. Chem.*, 1924, lix, 577.

[102] Johns, C. O., and Jones, D. B., *J. Biol. Chem.*, 1918, xxxvi, 491.

approximately half the protein content of the ration, and then determining the effect of additions of definite amino acids. The purpose of the gelatin addition is not particularly clear,[103] but its effect in profoundly modifying the amino acid mixture of the experimental ration and in impressing upon it its own amino acid deficiencies seems obvious. The further nutritive effects of additions of individual amino acids to the ration would presumably be determined more by the incomplete character of the amino acid mixture in gelatin itself than by the relative amino acid deficiencies of the protein studied. It is perhaps a matter of no surprise that, by the use of this method, cystine deficiencies have been discovered in the proteins of the Georgia velvet bean [103] and in edestin,[104] since this amino acid is totally absent from gelatin.

Sure's investigations of edestin that have led him to assume a deficiency in proline [101] are of particular importance, since if this assumption is correct, proline must be considered one of the amino acids that the body is unable to synthesize. Unfortunately, this study, even though it involves a comparison of the gain of rats with the amounts of proteins and amino acids consumed, falls far short of a demonstration. A ration containing 9 per cent of edestin, with cystine and lysine additions, was not definitely improved in its growth-promoting properties by an addition of proline (Charts I and II and Tables I and II of Sure's article). On the other hand, the growth of two rats on a 6 per cent edestin ration was distinctly slower than the growth of two other rats on the same ration plus proline (Charts III and IV and Tables III and IV of Sure's article), and the gain per gram of protein consumed was considerably less. But the two rats on the unsupplemented ration were females, and the two rats on the supplemented ration were males. The results of Hoagland and Snider [69] would indicate that such a difference between sexes, accompanied as they were by differences in food intake, may be obtained upon the same ration. It is further shown that two rats on a 6 per cent edestin ration, supplemented with cystine and lysine, did not respond to the addition of arginine, while two other rats on the same ration plus proline showed an acceleration of growth after a further addition of arginine to their food. The ratio of gain to protein consumed after the arginine additions was greater for the rats receiving proline than for those not receiving proline, but so was the food consumption and the rate of gain. It has already been shown in discussing the work of Hoagland and Snider that the gain per gram of protein consumed will increase for the same ration when the gain and food consumption in-

[103] Sure, B., *J. Biol. Chem.*, 1922, l, 103.
[104] Sure, B., *Amer. J. Physiol.*, 1922, lxi, 1.

crease, the increase in the ratio of gain to protein intake being a result of an increase in the proportion of the food that is available for growth. Under conditions, therefore, in which an increased ratio of one ration over that of another is accompanied by a markedly increased gain and food consumption, the difference in the protein economy of the gain cannot with certainty be interpreted as a response to an improvement in the quality of the protein. Thus, it must be concluded that Sure has not established the indispensability of proline in nutrition.

It is unfortunately necessary to emphasize in a review of this character that certain biological methods of experimentation inherently faulty and inefficient are being extensively used in the investigation of many problems connected with the nutritive value of proteins. The inevitable result has been that confusion prevails where orderly and consistent results might have been obtained with the expenditure of but little additional effort. The problem whether lactalbumin is a complete protein or a protein of superior biological value, only recently settled by Osborne and Mendel [105] * in the affirmative, is but another illustration of a case where the introduction of incompetent evidence has confused the issue. Perhaps, more than any other phase of the subject of this chapter, that of the supplementary relations existing among proteins has suffered the most from the employment of methods of research so designed that a clear-cut interpretation of their results is impossible.

THE SUPPLEMENTARY RELATIONS AMONG PROTEINS

It has been suggested that, for the purposes of practical dietetics, the different foods be classified according to the quality of their proteins into class A, B, C, etc. The difficulty encountered by this system is that the biological value of the proteins in mixtures of foods, such as are used in practical dietetics, is not necessarily the weighted mean of the biological values of the proteins of the individual foods. We may consider each food protein fed at a low level of intake as consisting of two fractions, one including the maximum amount of the several amino acids that can be used to replenish or enlarge the supply of nitrogenous substances in the tissues, the other including the remaining amount of the constituent amino acids destined to be deaminized, because it does not contain the complete assortment of amino acids essential for synthesis into complexes needed by the tissues. If two foods are fed together to a growing animal, those fractions of each that would otherwise be deaminized may together contain a complete assortment of amino acids,

[105] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1924, lix, 339.

* Attention may be called to the fact that Mitchell has demonstrated with rats ([19], p. 889) the attainment of nitrogen equilibrium with lactalbumin on low levels of intake.

permitting a part of the combined fractions to be used for synthetic purposes. In such a case, obviously the biological value of the mixture would be greater than the weighted mean of the biological values of each.

If the biological value of a protein is 70, then 30 per cent of its digestible fraction represents a mixture of amino acids of which it can only be said that it does not contain the amino acid limiting the utilization of the protein. It is this 30 per cent that is concerned in all supplementary relations with other proteins. It will, therefore, be referred to as "the supplementing fraction" of the protein. It must furnish all of the amino acids that are available for enhancing the nutritive value of other proteins, and it represents that part of the protein whose value in nutrition can itself be enhanced by the addition of amino acids from other sources.

From such considerations as these it may be inferred:

1. That proteins having the same amino acid deficiency will not exhibit supplementary relations, because the supplementing fraction of protein in each case will contain none of this particular indispensable amino acid. For example, milk and navy beans would not be expected to possess supplementary protein relations, because the limiting amino acid in each is cystine.

2. Conversely, proteins having different amino acid deficiencies will *always* exhibit some supplementary relation. The extent of the supplementing effect will depend upon the amino acid composition of the two supplementing fractions after their primary amino acid deficiencies have been mutually satisfied. It may be considered that the interaction between the two supplementing fractions will proceed until a common amino acid deficiency is reached in both.

3. Other things being equal, the greater the supplementing fraction of a protein mixture, the more general and the more marked will be its supplementary relations with other protein mixtures. A protein mixture, such as that occurring in eggs, possessing a high biological value of 95 and a low supplementing fraction of 5, would not be expected to exhibit as marked or general supplementary relations as that of beef, for example, with a biological value of 69 and a supplementing fraction equivalent to 31 per cent of its absorbed nitrogen.

In demonstrating a true supplementary relation between two proteins or between the mixed proteins of two foods, it seems essential to measure the nutritive value of each protein or of the mixed proteins of each food *separately,* and also in *combination,* the level of protein intake in all three tests being the same. Obviously, such a demonstration requires quantitative methods relating protein intake to growth secured.

nitrogen absorbed to total nitrogen retention, or some equivalent relation. The mere inspection of curves of growth obtained on rations limited only by their content of the individual proteins and of the mixed proteins is not sufficient to prove a supplementary relation, though it may establish a certain probability for the existence of such a relation. Also, an improvement in growth resulting from the substitution of a pure protein (casein, zein, gelatin) for some non-protein constituent of an experimental ration, is not a demonstration of a supplementary relation, in the sense here used, between the added protein *and the original protein compound* of the ration. Furthermore, the proof by quantitative methods that the substitution of small amounts of proteins inadequate for normal growth at a given level of protein intake by equal amounts of proteins of superior biological value, enhances greatly the growth-promoting value of the ration, is not a clear demonstration of a supplementing effect.

Maynard, Fronda and Chen [106] have demonstrated a supplementary relation between the proteins of corn and of rice bran. At an 8 per cent level, the gain per gram of protein consumed for corn proteins alone was 1.00 gram, for rice bran proteins alone 1.12 grams, and for a mixture of the two in the proportion of 2 to 1, 1.71 grams. At a 9 per cent level of intake, the values were, respectively, 1.18, 1.47 and 1.63 grams.* In this case, the mixture of proteins was evidently more efficient than the proteins of either food alone. An appreciable supplementary relation between peanut proteins and corn proteins was also revealed by these well-planned studies.

The value of gelatin, an incomplete protein by-product of the packing industry, as a supplement to cereal proteins is a question of practical importance to its proper evaluation as a food product. As the sole source of protein it will support neither growth nor maintenance, although a considerable fraction of its nitrogen may be used for the latter purpose, according to the investigations of McCollum and Steenbock,[17] Terroine, Fleuret, and Stricker,[107] and others. The question of its supplementing value has been approached from two angles. McCollum, Simmonds, and Pitz [108] have shown, in so far as growth curves alone are capable of showing, that additions of gelatin to whole oats and whole wheat, but not to corn, improve the value of their proteins, an effect assumed to be due to the lysine addition thus furnished. Downey, employing the same

[106] Maynard, L. A., Fronda, F. M., and Chen, T. C., *J. Biol. Chem.*, 1923, lv, 145.

* The differences in food intake in these experiments were generally slight and not such as to vitiate the conclusions drawn.

[107] Terroine, E. F., Fleuret, P., and Stricker, Th., *Compt. rend. acad. sci.*, 1923, clxxvii, 496; *Arch. intern. physiol.*, 1923, xxii, 43.

[108] McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1917, xxviii, 483; ibid., xxix, 341.

methods,[109] has obtained the same type of results with pearled barley, rolled oats, and, less clearly, with white wheat bread.

It may be expected that gelatin additions will improve the nutritive value of all protein mixtures except those having amino acid limitations corresponding to the amino acid deficiencies of gelatin itself, *i.e.*, tryptophane, tyrosine, and cystine. But such a relation is not the *mutual* supplementary relation under discussion. The question whether a mixture of oat proteins, for example, and gelatin has a higher growth-promoting value than that of oat proteins alone, or than that to be expected from the separate values of oat proteins and gelatin, is a different type of inquiry.

Osborne and Mendel [110] obtained results on one rat, using an oat protein concentrate containing about 80 per cent of the nitrogen of the oat kernel, indicating a distinct supplementary relation with gelatin. In 2 weeks of feeding, this rat required 9.2 grams of food per gram of gain on the oat concentrate ration containing 16.9 per cent of crude protein, while in the following 2 weeks, on a diet in which one fourth of the oat nitrogen was replaced by gelatin, the rat required only 3.8 grams of food per gram of gain. However, the growth-promoting value of this oat concentrate was evidently of a low order, since with three rats, only 0.65, 0.46, and 0.58 grams of gain were secured, in 2 weeks of feeding, per gram of protein consumed, and the total gain in the same time was only 6, 9 and 10 grams. The same investigators in later studies [36] have obtained much better growth with the protein of the entire oat kernel fed at much lower concentrations, so that it is perhaps fair to assume that whatever significance this one experiment possesses it cannot be assumed to apply to the entire mixed proteins of oats.

In 10 experiments on 5 rats, Mitchell [89] has been able to show that a mixture of whole oat proteins and gelatin in the ratio of 3 to 1 has a biological value of 43 at a 10 per cent level of feeding, while oat proteins alone at the same level have a value of 65. The individual values in these experiments were very concordant and the same rats were used in both tests; it is evident, therefore, that the mixture of oat proteins and gelatin was distinctly inferior in biological value to oat proteins alone. However, it seems difficult to determine whether a slight supplementary relation may not exist between oat proteins and gelatin, since the biological value of gelatin is hardly comparable with that of complete proteins. If it be assumed that, in the periods of oat-gelatin feeding, all of the gelatin nitrogen was absorbed and excreted in the urine, a biological value of

[109] Downey, T. B., *J. Met. Res.*, 1924, v, 145.
[110] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxiv, 521.

58 may be computed for the oat nitrogen. This is less than the value for oat nitrogen alone, so that no evidence is obtained on the basis of these assumptions that the quality of the oat proteins has been at all improved by admixture with gelatin.

Using the data obtained from nitrogen metabolism studies on pigs, Hart and Steenbock [85] have investigated the protein efficiency of mixtures of corn and milk in various proportions, with results summarized in Table 50. The marked rise in the percentage of the nitrogen intake retained by the pigs as the proportion of milk protein is increased from 20.7 to 28.5 per cent is due largely to a marked improvement in the digestibility of the nitrogen of the ration. A less marked increase is indicated by the percentages of absorbed nitrogen retained, while the percentages of absorbed nitrogen used for both maintenance and growth (the biological values) show a linear increase from the first to the last group of pigs, with the exception of the next to last group on a ration containing 20.7 per cent of milk nitrogen. From the computations in the last column it is apparent that if corn had been fed alone its proteins would have shown an efficiency of a little over 60 per cent. With the fifth group of pigs receiving about 30 per cent of milk proteins, if no supplementary relation existed and if the efficiency of the milk proteins is taken as 100 per cent, it may be computed that the efficiency of the combined proteins in this ration would be about 72 per cent. The difference between 72 and 87, the efficiency actually attained, demonstrates a supplementary relation between the proteins of the two feeds.

A similar marked supplementary relation between oat proteins and milk proteins is indicated by other metabolism studies on swine reported by Hart and Steenbock.[88] For a mixture of these proteins in the approximate proportion of 3 to 1, biological values of 81 and 82 may be computed for two pigs from these data, on the assumption of average values for the endogenous nitrogen.

Mitchell [89] has investigated the supplementary relations between corn and milk proteins in the proportion of 3 to 1 at a 10 per cent level of intake, using rats as subjects. Corn proteins alone exhibited an average biological value of 61.3 in these experiments, milk proteins alone a value of 84.7, and the combination a value of 75.7. The mean of the first two values weighted 3 and 1, respectively, is 67.2. Therefore, the difference between this mean and the value actually found, 75.7, indicates clearly that the amino acid deficiencies of corn proteins are supplemented by the excess amino acids of milk proteins.

A distinct supplementary relation was also demonstrated between corn proteins and the proteins of tankage. In farm practice, good results

are obtained in the growth and fattening of swine by feeding a mixture of corn and tankage.* The value of the latter feed in supplementing corn is commonly supposed to be twofold: first, in contributing an abundance of calcium and phosphorus; and, second, in contributing an abundance of protein, of which the best grades of tankage contain 50 to 60 per cent. However, no information is available as to whether the value of tankage as a protein supplement depends simply on the intrinsic value of its proteins or on their supplementary relations with the proteins of corn. In another paper,[21] duplicate results on each of three rats were reported relative to the biological value of tankage proteins themselves fed at a 10 per cent level. This value seems to be exceptionally low, averaging 31.5. However, when tankage and corn are mixed in such proportions that the protein of the mixture is derived equally from the two feeds, the biological value of the combined proteins is fully equal to that of corn proteins and possibly is even higher. The average value obtained was 62, very close to the average value obtained for corn in these investigations, *i.e.*, 60. This, of course, would not be the case unless a distinct supplementary relation existed between the proteins of corn and the proteins of tankage. It would seem, therefore, that the value of tankage as a protein supplement for corn depends on a distinct increase in the protein content of the ration, but not upon any appreciable increase in the biological value of the protein component of the ration.

The supplementary relation between the proteins of corn and of tankage thus indicated by experiments on rats have been confirmed by Mitchell and Kick [85a] on 8 pigs. At an 8 per cent level of intake the proteins of corn gave an average biological value of 54, those of tankage 42, and those of a mixture in the ratio of 2 to 1, an average value of 61. These results point to a more pronounced supplementing effect than those obtained with rats.

Many other instances have been reported of marked supplementary relations between the proteins of animal foods and those of vegetable foods, particularly cereal foods. Osborne and Mendel [16] observed that when egg, milk or meat powders were combined with white flour in such proportion that one-third of the total protein in the ration was furnished by the animal product, the mixture of proteins induced much more rapid growth in rats than flour protein alone, and about three times the gain in weight per gram of protein consumed.

* The latter feed contains packing house refuse, including meat scraps, condemned meat, bones, blood, and viscera, that has been submitted to prolonged treatment with steam under pressure for the removal of most of its grease. The product is then dried and finely ground. In regulating the composition of tankage, attention is paid only to total nitrogen, the content of which is varied by varying the proportion of blood and of the evaporated liquid ("stick") obtained in the drying of previous batches of tankage.

Mitchell and Carman [77] have investigated the biological values of combinations of animal foods and white flour containing flour proteins and animal proteins in the ratio of 2 to 1. Such a mixture of white flour and egg proteins possessed a biological value of 75. If no supplementary relation existed between these two sources of nitrogen, the value expected from the mixture, computed from the biological values of the nitrogen of the individual foods at the same level of intake, would be 66. The difference between 66 and 75 may, therefore, be taken to indicate and to measure a mutual supplementary relation.

Only a slight supplementary relation was found between the proteins of egg white and of white flour, the observed biological value of the mixture being 66 and the expected value 64. A similar mixture containing milk proteins in place of egg white proteins was found to possess a biological value of 71, as compared with an expected value of 62. A particularly fibrous sample of veal, giving a biological value of 62, when combined with twice as much white flour proteins, themselves possessing a biological value of 52, still showed a biological value of 62. Similarly, a sample of beef round, with a biological value of 69, when combined with white flour in the same manner, gave a protein mixture with a biological value of 73, as compared with an expected value of 60. Milk and cocoa proteins do not appear to possess any supplementary effect upon each other.[79]

The marked supplementary relation between meat proteins and white flour proteins has been confirmed by Hoagland and Snider,[72] in feeding experiments on rats, and extended to other cereal flours. When beef protein was mixed in equal proportions with vegetable proteins, the rations containing whole wheat, bolted wheat flour, corn meal, oatmeal, and rice showed approximately the same efficiency in promoting growth, as measured by the gain per gram of protein consumed. Each of these mixtures also had approximately the same value for promoting growth as a ration containing the same percentage of beef protein alone.

On the other hand, rations containing equal parts of beef and navy bean or potato nitrogen gave much lower values, but the food consumption and the rate of gain were also much lower, so that the significance of the difference in the protein economy of the gains is not clear. If, as Hoagland and Snider have so clearly shown,[69] marked differences in rate of gain and in food consumption between male and female rats *on the same rations* induce marked differences in the gain per gram of protein consumed, similar marked differences in the gain per gram of protein consumed between groups of male rats *on different rations,* if accompanied by corresponding differences in rate of gain and in food

consumption, *may* be explainable in the same way, without reference to the difference in composition of the rations.

The same difficulty in interpreting the results of this method of measuring the nutritive value of protein applies to the data obtained by Hoagland and Snider with beef, pork, and lamb as the sole source of dietary protein, as compared with data obtained with rations in which wheat, wheat flour, oatmeal and navy beans constituted the sole source of protein. The former rations induced similar large gains per gram of protein consumed, while the latter induced much smaller gains on the same basis, particularly the wheat flour and navy bean rations. But these smaller gains per gram of protein consumed were accompanied by smaller *rates* of gain and smaller *rates* of food consumption.

It would appear that differences in the nutritive value of proteins can be demonstrated by this method only when the *rates* of gain on the different rations are the same. This essential condition to the clear interpretation of experimental results may be left to chance by *ad libitum* feeding, as is the usual practice, or may be definitely assured by controlled feeding, such that the rate of gain of rats on the more palatable or the more efficient ration is restricted to that secured on the less successful ration by restricting the food consumption.

When beef protein constituted but one-third of the mixed protein in the ration in the experiments of Hoagland and Snider, the combinations with oatmeal and corn meal induced gains per gram of protein consumed similar to those obtained on beef protein alone at the same level of intake, slightly more favorable results being obtained with oatmeal than with cornmeal. The combinations of beef and wheat, and beef and wheat flour induced much smaller rates of gain as well as less economical gains with reference to the total protein consumed.

The supplementary relations among a large variety of proteins from different sources, indicated by the extensive experiments of McCollum, Simmonds, and Parsons,[44, 8] cannot be profitably discussed for reasons already set forth. To any one holding the views advocated in this chapter concerning the technical requisites of a determination of the nutritive value of any definite source of protein, these extensive and frequently cited investigations present only a confusing mass of evidence, the interpretation of which in terms of the protein mixtures fed seems utterly impossible. The interpretation that is given to it by the authors of the reports themselves is burdened with assumptions of the toxicity of natural foods (millet, pea) hitherto unsuspected, and of intricate interrelations among nutritive requirements that can hardly be accepted on the evidence offered. Though a large number of rats were used, the

rations tested were also numerous, and so many factors aside from growth contributed to the conclusions drawn that the evidence on any one point frequently appears to be statistically insufficient. The reproductive records in particular varied greatly, and with only 2 females to a group ordinarily, their proper interpretation is obscure in many cases. If these experiments represent, as is claimed, the most refined method available for the study of the supplementary relations existing among the proteins of foods, then the conceptions upon which it is based are so far in advance of those developed in this chapter that the writers feel totally incapable of assessing their true value. If this situation actually exists, practically all of the experimental data that have been accumulated up to the present writing by other investigators are of doubtful significance.

While many instances of supplementary relations between animal and vegetable proteins have been indicated or demonstrated by feeding experiments or metabolism studies, few instances of an appreciable supplementary effect of the proteins of one vegetable product upon those of another have been reported in so far as the writers are aware, aside from the published investigations of McCollum, Simmonds and Parsons. Nevens has reported the results of combining in all possible ways the proteins of corn, cottonseed meal, and alfalfa [74]; for only one combination, *i.e.*, that of cottonseed meal and alfalfa, was any such effect indicated, and the effect was slight. In another report from the same laboratory,[75] Mitchell and Villegas have shown that no supplementary relations exist between the proteins of corn and of coconut meal, though between the proteins of corn and soybeans there may be a slight supplementary effect.

Maynard, Fronda and Chen [106] have also failed to detect any considerable supplementary relation between the proteins of corn and those of soybeans. Combinations of corn and linseed meal, and corn and cottonseed meal resulted in protein mixtures but little if any superior in nutritive value, to those of corn alone. On the other hand, the proteins of peanut oil meal and of rice bran appeared definitely to supplement the proteins of corn and to yield mixtures of proteins appreciably higher in growth-promoting value than corn proteins themselves.

The high supplemental value of the proteins of meat and milk when fed in combinations with cereal proteins is of great practical importance in human nutrition. From the results of a large number of dietary studies made throughout this country by the U. S. Department of Agriculture, Langworthy [111] estimates that some 43 per cent of the protein of the

[111] Langworthy, C. F., *U. S. Dept. Agri., Off. Exper. Sta.*, Circ. 110, 1911.

American diet is derived from milled cereals. According to Pearl [112, p. 226] the average percentage distribution of the dietary protein consumed in this country from 1911 to 1917 among the different classes of foods was as follows: grains 36, meats 26, dairy products 20, poultry and eggs 7, vegetables 6, fish 2, and all other foods 3. Therefore, the importance of animal proteins in the American diet appears to reside as much in the extent to which they improve the utilization of inferior cereal proteins as in their own nutritive excellence.

The supplementary relations existing among food proteins represent a field of research that has hardly been opened up. It is closely related to such problems as the determination of the indispensable amino acids among those found in the protein molecule, and the determination of the primary, secondary and subsequent amino acid deficiencies in the protein mixtures of foods. With information supplied by experiments concerned with these problems, it should be possible to investigate the supplementary relations among food proteins by a more intelligent and effective method than that involving promiscuous combinations of foods.

THE BIOLOGICAL VALUE OF PROTEINS FOR MILK PRODUCTION

It would seem that the most profitable experimentation on the relative values of different proteins for milk production must be confined to those species of animals from which the milk can be collected, measured and analyzed. Otherwise the milk production must be measured by the growth of the young, which would be affected by variations in the composition of the milk as well as variations in the rate of its secretion. The interpretation of the growth secured in terms of the amount of protein consumed by the mother would be difficult in such a case.

The work of Hart and Humphrey [113] on the effect of the quality of food proteins on milk production in dairy cows illustrates well the difficulties involved in this type of research, even when the milk can be collected, measured and analyzed. With immature or pregnant cows, the efficiency of the protein intake, however measured, relates to maintenance, growth and milk production. If the cows are in negative nitrogen equilibrium, tissue proteins are undoubtedly being used for milk production and are, in all probability, supplementing the food proteins for that purpose. If considerable positive nitrogen balances are obtained, there is no assurance but that protein is being consumed in excess of requirements, thus depressing any calculated efficiency of the food pro-

[112] Pearl, R., "The Nation's Food: A Statistical Study of a Physiological and Social Problem." Phila. and London. 1920.

[113] Hart, E. B., and Humphrey, G. C., *J. Biol. Chem.*, 1912-13, xiii, 133; *ibid.*, 1914, xix, 127; *ibid.*, 1915, xxi, 239; *ibid.*, 1916, xxvi, 457; *ibid.*, 1917, xxxi, 445; *ibid.*, 1918, xxv, 367.

teins, particularly of the most superior combinations. It is significant that in the only experiment of Hart and Humphrey in which positive balances were obtained generally, the differences between the calculated efficiencies of the proteins of the feeds tested were least.*

While the experiments of Hart and Humphrey on dairy cows, and of Hoobler [114] on women, indicate the superior value of milk proteins for milk production, they also indicate that the difficulties attending this type of investigation have not been removed sufficiently to permit the calculation of protein efficiencies that can readily be interpreted.

Forbes and Swift [115] have calculated relations between the nitrogen stored in the milk and in body tissue and the nitrogen intake, or the nitrogen intake minus an estimate of the nitrogen required for maintenance, in 45 balance periods on a number of dairy cows subsisting upon good dairy rations. The data were obtained from experiments not planned with the idea of determining the maximum efficiency of utilization of the dietary protein, and naturally the results obtained cannot be so interpreted. It seems that more significant utilization percentages might be obtained involving the digestible nitrogen rather than the nitrogen intake, while the significance of the difference between the *total* nitrogen intake and what appears to be an estimate of the *digestible* nitrogen required for maintenance is somewhat obscure.

An attempt to compare the values of peanut meal, cottonseed meal, and soybean meal as sources of protein for milk production by the use of nitrogen metabolism studies and calculations of biological values, has been reported by Holdaway, Ellett and Harris.[116] Of these nitrogenous concentrates, peanut meal was found to be the most efficient with an average biological value of 84. The average values of cottonseed meal and soybean meal were 78 and 77, respectively. These values are probably appreciably higher than the actual values, because, in the absence of information concerning the endogenous urinary nitrogen, estimates of the digestible nitrogen required for maintenance were substituted. In these experiments, considerable positive or negative nitrogen balances

* In *Memoir 113* of the Cornell University Agricultural Experiment Station, issued in March, 1928, Maynard, Miller, and Krauss have reported the results of experiments on dairy cows designed to compare the efficiency of clover and timothy rations in covering the protein requirements for maintenance and milk production. It was found impossible, however, to prepare practicable rations in which these roughages would furnish even as much as 50 per cent of the protein intake, and, with the timothy rations, a much smaller proportion of the total nitrogen was contained in the hay. The percentage utilization of the absorbed nitrogen is computed in the same manner as in the experiments of Hart and Humphrey, and thus neglects the nitrogen used for maintenance, which is *not* represented in the nitrogen balance, as the authors appear to believe. For the clover ration, an average percentage utilization of 54.8 ± 1.7 was obtained, and for the timothy ration an average percentage of 58.0 ± 2.0. This close agreement is perhaps not surprising, since, except for the hays, the rations generally contained the same feeds, though in different proportions.

[114] Hoobler, B. R., *Amer. J. Dis. Children*, 1917 xiv, 105.

[115] Forbes, E. B., and Swift, R. W., *J. Dairy Sci.*, 1925, viii, 15.

[116] Holdaway, C. W., Ellett, W. B., and Harris, W. G., *Va. Agr. Exp. Sta. Tech. Bull.* 28, 1925.

frequently prevailed; the extent of their disturbing effect on the significance of the estimates of biological value cannot be told.

In nitrogen metabolism studies on mature dairy cows undertaken for the determination of the biological values of feed protein, it would appear that a careful adjustment of the protein content of the rations and of the nitrogen intake should be made, so that the animal is maintained in slight negative nitrogen balance throughout; also, that comparisons of different feed proteins should be made at the same level of intake, and with either the same cows in comparable stages of lactation or with different cows of approximately the same productive capacity. If immature dairy cows are used, slight positive balances will not, of course, have the same vitiating effect on the significance of the estimated biological values as if mature cows are used.

THE PROTEIN VALUES OF FOODS

The biological values of food proteins, like the coefficients of digestibility, measure the utilization of protein, but do not in themselves measure the protein values of the food. The protein value of a food depends as much upon the content of protein as upon the extent of its utilization in digestion and in anabolism. It therefore appears to be of great practical importance to combine the content of protein, with its percentage utilization in digestion and in anabolism, in estimating how much is ultimately available in covering the protein requirements of maintenance and growth. The losses of body nitrogen in the feces consequent upon the digestion of the food should be considered in this connection.

A method of obtaining numerical measures of the protein values of foods, involving all of these considerations, has been proposed by Mitchell,[117] and results illustrating the method will be found in Table 52.

To illustrate the method and to understand its significance, it is necessary to follow in detail the calculation for one food:

Taking beef round as an example, according to average analyses lean beef round contains 21.3 per cent of protein on the fresh basis and 71.0 per cent on the dry basis. The digestibility of this protein, making allowance for the metabolic nitrogen of the feces, is 96 per cent according to a number of experiments on rats. Hence, on the fresh basis, beef round contains 20.4 per cent of digestible protein, and on the dry basis, 68.2 per cent. However, all of the absorbed protein is not available in anabolism, mainly because of some disproportion among the constituent amino acids as compared with the body's requirements. In rat experi-

[117] Mitchell, H. H., *J. Home Economics*, 1927, xix, 122.

TABLE 52. *Protein Values of Foods for Maintenance and Growth: Level of Protein Feeding, 8 to 10 Per Cent.*

Food	Water Content[1]	Protein Content[1] On Fresh Basis	Protein Content[1] On Dry Basis	Quality of Protein Digestibility (corr.)	Quality of Protein Biological Value	Metabolic Protein in Feces[2] On Fresh Food Basis	Metabolic Protein in Feces[2] On Dry Food Basis	Protein Value of Food On Fresh Basis	Protein Value of Food On Dry Basis
	%	%	%	%	%	%	%	%	%
Whole egg[3] ..	73.2	13.2	49.3	100	94	0.4	1.4	12.0	44.9
Milk	87.0	3.3	25.4	100	85	0.2	1.4	2.6	20.2
Egg white[3] ..	86.2	12.3	89.1	100	83	0.2	1.4	10.0	72.6
Beef liver ...	71.2	20.4	70.8	90	77	0.4	1.4	14.9	51.1
Beef kidney ..	76.7	16.6	71.3	99	77	0.3	1.4	12.3	52.8
Beef heart ...	62.6	16.0	42.8	100	74	0.5	1.4	11.3	30.3
Beef round ..	70.0	21.3	71.0	96	69	0.4	1.4	13.7	45.7
Pork ham ...	60.0	25.0	62.5	100	74	0.6	1.4	17.9	44.8
Veal[4]	73.4	20.7	78.0	100	62	0.4	1.4	12.4	47.0
Rolled oats ..	7.7	16.7	18.1	90	65	1.3	1.4	9.8	10.6
Whole wheat .	11.4	13.8	15.6	91	67	1.3	1.4	7.1	8.1
White flour ..	12.8	10.8	12.4	100	52	1.3	1.4	4.3	5.0
Whole corn ..	10.3	7.5	8.4	95	60	1.3	1.4	3.0	3.5
Potato	78.3	2.2	10.1	78	67	0.3	1.4	0.8	3.9
Navy beans[3]..	12.6	22.5	25.7	76	38	1.3	1.4	4.2	6.0
Cocoa	4.6	21.6	22.6	38	37	1.4	1.4	1.6	1.8
Chocolate	5.9	12.9	13.7	38	37	1.4	1.4	0.4	0.6

[1] Average analyses taken, as far as possible, from *Bull. 28 (revised), Office of Experiment Stations, U. S. Dept. Agr.*

[2] The metabolic nitrogen in the feces is assumed to equal 0.23 gram per 100 grams of dry matter of food. See *Bull. Natl. Research Council,* 1926, xi, pt. 1, no. 55, p. 23.

[3] Cooked.

[4] The cut tested was not recorded. It proved to be very fibrous. Analysis for shoulder cut assumed.

ments we have found an average of 69 per cent of the absorbed protein available for the requirements of maintenance and growth at a level of 8 to 10 per cent of protein in the diet. This 69 per cent is the "biological value" of beef round protein, a term introduced and defined by Thomas. Hence, what might be called the "net protein" in beef round is $20.4 \times 0.69 = 14.1$ per cent on the fresh basis, and $68.2 \times 0.69 = 47.1$ per cent on the dry basis.

But this cannot be considered the final protein value of the food, since in the digestion of food, protein (nitrogen) is inevitably lost from the body in the feces. This is the so-called "metabolic nitrogen" of the feces. As established elsewhere,[1] it appears to be a wastage of nitrogen associated with digestion rather than with metabolism. It should, therefore, be charged against the food, and in particular against the protein of the food. It may be questioned why it was not charged against the food in computing the absorbed protein, by using the coefficient of *apparent* digestibility rather than the coefficient of *actual* digestibility. The answer is that the biological value applies to the actual absorbed

protein, not to the fictitious figure obtained by the use of the ordinary coefficient of digestibility uncorrected for the metabolic nitrogen in the feces. Hence, some other method of properly allowing for this wastage of nitrogen must be applied.

From a study of a large number of experiments in which different species of animals were fed nitrogen-free diets, the rather surprising fact was discovered [1] that the metabolic nitrogen in the feces bears a rather constant relation to the dry matter consumed, except in cases where the food contained considerable amounts of crude fiber. This relation was found to be approximately 0.2 gram of metabolic nitrogen per 100 grams of dry matter. Averaging a considerable number of reported experiments on human subjects gave a value of 0.23 gram. Hence, in the calculations in the table, this value, equivalent to 1.4 grams of protein per 100 grams of dry matter, or 1.4 per cent is assumed. On the fresh basis, for beef round, this value becomes $1.4 \times 0.30 = 0.4$ per cent. The final protein values of beef round are obtained by deducting these percentages from the percentages of "net protein," giving $14.1 - 0.4 = 13.7$ per cent on the fresh basis, and $47.1 - 1.4 = 45.7$ per cent on the dry basis. These values presumably give the maximum percentage of protein ($N \times 6.25$) in the food which is actually available for the maintenance and growth of the nitrogenous constituents of the tissues.

The calculations in the table represent a new method of evaluating foods with respect to the nutritive value of their protein ($N \times 6.25$), a method that takes cognizance of both the quality and the quantity of the protein ($N \times 6.25$) in foods.

The protein values summarized in the last two columns of the table afford a precise statement of the known superiority of animal foods over vegetable foods as sources of protein in the diet. Even such vegetable foods as dried (navy) beans and cocoa, which are relatively rich in crude protein, are unimportant as sources of protein in nutrition, because of the enormous losses of nitrogen in the course of their utilization in digestion, or in metabolism, or in both. Their high content of protein is deceptive, and the conclusion that such foods are "meat substitutes," though frequently stated, must be considered erroneous. Of particular significance are the data relating to white flour, since this food enters so largely into the diet of all classes of the American people. With an initial content of 10.8 per cent of protein, its final protein value becomes 4.3 per cent on the flour as purchased, mainly because of a loss of 48 per cent in metabolism. The marked superiority of whole wheat over white flour and the distinct superiority of whole oats over whole wheat as sources of protein are worthy of note. The proteins of de-

germinated corn meal would probably possess a distinctly lower biological value than the proteins of whole corn.

Among the animal foods it is evident that meats and meat products are preëminent as sources of protein. Although the biological values of animal tissue proteins (nitrogen) are appreciably lower than those of eggs or of milk, the higher content of protein in animal tissues, either on the fresh or the dry basis, offsets or more than offsets their greater losses in metabolism.

The nitrogenous compounds in different cuts of the same kind of meat may differ greatly in chemical characteristics, indicating in particular a different proportion of connective tissue. The varying content of connective tissue in different cuts of meat modifies the biological value of their nitrogen in such a way that the greater the proportion of connective tissue the smaller is the biological value. The low value for veal reported in the table is undoubtedly associated with the high connective-tissue content of the sample tested. The relatively high biological values for liver, kidney and heart may well be associated with a relatively low content of connective tissue. From such considerations it may be concluded that the cheaper cuts of meat, known to contain relatively much connective tissue, are lower in nutritive value with reference to protein than the higher priced cuts. The close association between the toughness of meat and its content of connective tissue, in connection with the facts just discussed, would appear to justify the statement that the toughness of raw meat is a rough indication of the biological value of its protein.

It is a singular fact that in the preparation of animal carcasses for human consumption, as in the preparation of the cereal grains, the portions of the product in least demand have been found to possess the greatest dietetic value with reference to certain nutrients. The superiority of many of the visceral organs of animals over the muscle meats as sources of vitamins is analogous to the superiority of the germ and outer layers of the cereal grains over the endosperm, of which the cereal flours consist. With seeds this superiority of the "offal" extends to the character of the proteins and to the mineral content. That the proteins of liver, heart and kidney are somewhat superior in quality to the proteins of carcass meat is indicated by the biological values reported in the table. In the same connection it may be mentioned that Forbes and Swift [118] have recently reported that the iron content of these organs is much higher than that of muscle meats, while Robscheit-Robbins and Whipple's [119] experiments have indicated the marked superiority of liver

[118] Forbes, E. B., and Swift, R. W., *J. Biol. Chem.*, 1926, lxvii, 517.
[119] Robscheit-Robbins, F. S., and Whipple, G. H., *Amer. J. Physiol.*, 1925, lxxii, 408.

over all other foods, including meats, in the promotion of blood regeneration after hemorrhage. Evidently the food value of the edible animal viscera is high and unique in several respects, and their consumption should be encouraged by dietitians and physicians.

The biological values reported in the table may be considerably altered by an alteration in the level of protein feeding. A diet containing 8 to 10 per cent of protein is considered a low-protein diet in human nutrition; according to Sherman [120] a maintenance diet for a man at moderate activity should contain 6 per cent of its available calories in the form of protein. As the level of protein decreases from 10 to 5 per cent, which is near the maintenance level for the rat also, the utilization of proteins in metabolism will in general increase. For example, the biological value of milk proteins will increase from 85 to 93, that of corn proteins from 60 to 72, and that of oat proteins from 65 to 79. At the 5 per cent level, casein has been found to have a biological value of 71, rice proteins a value of 86, and yeast proteins a value of 85. If the level of protein is increased, a decrease in biological value occurs. For example, in one experiment the value for beef liver was found to be 82 at an 8 per cent level and only 58 at a 16 per cent level. It appears that, with respect to protein as well as with respect to energy, the animal body economizes at low levels of intake and becomes increasingly extravagant at higher levels. One practical conclusion from this situation is that, in adult nutrition, where the protein requirement is low, the quality of the protein fed, even in low-protein diets, is not a matter of concern. This is the conclusion to which Sherman has come from a study of a large number of experiments concerned with the determination of the minimum protein requirement of men, while Rose and MacLeod [29] have reported essentially the same results.

THE PHYSIOLOGICAL EFFECTS OF PROTEIN

The possible physiological effects of the ingestion of amounts of protein above the minimum requirements of animals, particularly of man, has occasioned a large amount of discussion and of experimental investigation. The question at issue is whether proteins are nutrients that should be partaken of sparingly, because their digestion and metabolism exposes the cells to the action of metabolites that may disturb their normal functioning, and imposes a burden upon the kidneys in eliminating them from the body.

Probably the first investigation apparently affording experimental verification for the belief that protein is capable of exerting such dele-

[120] Sherman, H. C., *J. Biol. Chem.*, 1920, xli, 97.

terious effects, was that of Chittenden.[121] This investigation involved a large number of observations upon three groups of human subjects subsisting upon low-protein diets for several consecutive months. It was found that the marked reduction in the allowance of protein prescribed by current dietary standards not only failed to induce malnutrition, but actually appeared to promote the health and well-being of the subjects. Chittenden, on the basis of these results, eulogized the low-protein régime, and enunciated a doctrine of physiological economy in nutrition that has found its way, in one form or another, into several systems of dietetics. The later studies of Hindhede [122] have established the adequacy, in adult nutrition at least, of protein intakes even smaller than those investigated by Chittenden.

However, in the now classical experiments of Chittenden the absence of control groups of subjects maintained upon the usual protein intake but otherwise subjected to the same routine of life as the other groups, must be keenly felt by the critical appraiser of their significance, and renders questionable the deduction that the observed benefits of the experimental conditions imposed were due solely, if at all, to the reduction in protein intake. There are communities and races of men that have subsisted for generations on small amounts of protein comparable to that advocated by Chittenden and Hindhede, but their physical, intellectual and industrial characteristics are not such as to inspire confidence in the wisdom of their habits or any desire to follow in their footsteps. Among such communities, the low-protein diets in vogue are not a matter of choice but a matter of necessity, due to a food supply restricted by climate, geographical location, or industrial backwardness.

It has seemed a reasonable proposition to many physiologists that the elimination of the nitrogenous metabolites of protein should constitute an onerous burden on the kidneys, and that the continuous ingestion of immoderate amounts of protein would ultimately prove harmful to these essential organs. In confirmation of this proposition, Newburgh in 1919 [123] reported experimental observations on rabbits fed high-protein diets. Acute, subacute or chronic nephritis was noted in all cases and was attributed to the excessive protein intake. Again in 1923, Newburgh and Clarkson [124] described dilatation of the urinary tubules and some scarring of the glomeruli in the kidneys of rabbits subsisting for 6 to 12

[121] Chittenden, R. H., "Physiological Economy in Nutrition." New York. 1904.
[122] Hinhede, M., *Skand. Arch. Physiol.*, 1913, xxx, 97; 1914, xxxi, 259; "Protein and Nutrition." London. 1913.
[123] Newburgh, L. H., *Arch. Intern. Med.*, 1919, xxiv, 359.
[124] Newburgh, L. H., and Clarkson, S., *Arch. Intern. Med.*, 1923, xxxii, 850.

months on diets containing 27 or 36 per cent of protein. Squier and Newburgh,[125] in experiments on human subjects, observed the appearance of red blood corpuscles in the urine and the production of albuminuria or the aggravation of an existing albuminuria in patients with varying degrees of kidney damage following forced high-protein feeding. Furthermore, forced high-protein feeding with normal men invariably induced the appearance of red blood corpuscles in the urine.

In an attempt to explain the renal injuries apparently induced by high-protein feeding, Newburgh and Marsh [126] determined the effects on the kidneys of rabbits and puppies of the intravenous injection of individual amino acids. The intravenous administration of alanine, leucine, glycine, phenylalanine, and glutamic acid gave no evidence of renal injury in doses as large as 2 grams per kgm. of body weight. Arginine and aspartic acid were mildly nephrotoxic, while serious kidney damage was produced by lysine, histidine, tyrosine, tryptophane and cystine. Evidence of the occurrence of kidney injury was obtained by microscopic examination of the urine and of the kidney itself. Urea was without effect upon the kidney. Lewis [127] had previously reported the production of serious renal injury in rabbits by the oral administration of the sodium salt of cystine in doses of 0.5 to 1.0 gram per kgm. of weight.

From these investigations, Newburgh is inclined to the opinion that dietary protein is one of several contributing factors in the etiology of human chronic nephritis.[128]

The feeding experiments of Newburgh and his associates with rabbits may be criticized because none of the experimental diets used were demonstrably complete in other dietary factors than protein, and several of the diets were quite obviously incomplete. Hence, pathological conditions developing in experimental animals after continued subsistence upon such diets may as reasonably be attributed to dietary deficiencies as to the excessive protein intake. The validity of this criticism is fully supported by the investigations of Maclean, Smith and Urquhart,[129] who showed that renal injury could be readily induced in rabbits on either low- or high-protein dietaries that were obviously incomplete in other respects, and that on such diets renal injury could be either

[125] Squier, T. L., and Newburgh, L. H. *Arch. Intern. Med.*, 1921, xxviii, 1.

[126] Newburgh, L. H., and Marsh, P. L., *Arch. Intern. Med.*, 1925, xxxvi, 682. Further work on the toxic action of cystine on the kidney and the liver has been reported by Curtis and Newburgh (*Arch. Intern. Med.*, 1927, xxxix, 817, 828).

[127] Lewis, H. B., Proc. Amer. Soc. Biol. Chem., *J. Biol. Chem.*, 1925, xliii, p. xx.

[128] Newburgh, L. H., *Medicine*, 1923 ii, 77. Newburgh, Marsh, P. L., Clarkson, S., and Curtis, A. C., *J. Amer. Med. Assoc.*, 1925, II, lxxxv, 1703.

[129] Maclean, H., Smith, J. F., and Urquhart, A. L., *Brit. J. Exper. Path.*, 1926, vii, 360. The negative results of Anderson (*Arch. Intern. Med.*, 1926, xxxvii, 297, 313), who fed rabbits for "about a year" on a beef protein ration containing 30.5 per cent of protein, may also be cited.

cured or prevented by the daily consumption of cabbage leaves.* These experiments show that rabbits may subsist for over five months on diets containing 60 per cent of protein (largely casein and gelatin) with no evidence of nephritis or of chronic inflammatory changes of any description. Hence, the conclusion appears justified that the nephritis observed in the experiments of Newburgh and associates, in rabbits maintained on dietaries containing much smaller percentages of protein, was probably unrelated to the protein consumed.

From numerous feeding experiments on rats it also appears extremely probable that excessive protein ingestion bears no relation to kidney disease. It is true that Polvogt, McCollum, and Simmonds [130] have observed evidences of kidney involvement (hypertrophy, intense congestion, hyaline casts) in rats raised on adequate diets containing 31 to 41 per cent of protein, but the significance of this purely histological evidence is greatly discounted by the excellent growth and high fertility induced by the experimental diets. The infant mortality was low and, from observations extending over five generations raised upon these diets, no deterioration in nutritive condition was observed. Under these conditions, it may be doubted whether the abnormal appearance of the kidneys indicated disturbance in function.

Evans and Risley [131] have also reported nephritic changes in rats fed on high-protein rations for prolonged periods (7 to 15 months). However, Jackson and Riggs [132] have fed rats for as long as 20 months on diets containing 76 per cent of protein (casein), but have been unable to produce in such animals any recognizable nephritis, although considerable hypertrophy of the kidneys (24%) developed. Periodical examination of the urine showed persistent slight albuminuria in the control as well as in the high-protein rats. Drummond, Crowden, and Hill [133] and Reader and Drummond [134] have fed rats and cats on diets containing as high as 90 per cent of protein (casein), and although adult size could not be attained on such one-sided diets, no evidence of kidney damage was observed after 100 to 120 days of feeding. The only effect of the abnormal excretory activity induced by these very high-protein

* Possibly the high urea content of the blood and tissues of guinea pigs in which scurvy has been produced by oat feeding as observed by Lewis and Karr (*J. Biol. Chem.*, 1916, xxviii,) was the result of a nephritic condition of the kidneys, analogous to that produced in the rabbit by Maclean, Smith and Urquhart on an oat and bran diet with no greens.

[130] Polvogt, L. M., McCollum, E. V., and Simmonds, N., *Bull. Johns Hopkins Hosp.*, 1923, xxxiv, 168. Nuzum (*Arch. Intern. Med.*, 1927, xl, 364) has also produced distinct evidences of kidney damage in rabbits after 24 months on the Polvogt, McCollum, Simmonds diet.

[131] Evans, N., and Risley, E. H., *Cal. and Western Med.*, 1925, xxiii, 437.

[132] Jackson, H., and Riggs, M. D., *J. Biol. Chem.*, 1926, lxvii, 101. However, Jackson and Moore (*J. Clin. Invest.*, 1928, v, 415) later noted that high-protein diets produced kidney damage to the remaining kidney of unilaterally nephrectonized rats. Individual susceptibility and age determined the degree of this damage.

[133] Drummond, J. C., Crowden, G. P., and Hill, E. L. G., *J. Physiol.*, 1922, lvi, 413.

[134] Reader, V. B., and Drummond, J. C., *J. Physiol.*, 1924-25, lix, 472; *Biochem. J.*, 1926, xx, 1256.

diets was a marked enlargement of the kidneys. Kennedy [135] has confirmed these results of Drummond and coworkers.

Miller [136] has fed different groups of rats diets containing varying percentages of protein (casein and various vegetable proteins) up to 40 per cent. These diets were otherwise well balanced. Although the higher protein diets induced kidney hypertrophy, microscopic examination of the kidneys revealed no evidence of kidney damage, even in rats from which one kidney had been removed.

Addis, MacKay and MacKay [137] have raised rats from 30 days to one year of age on diets containing 70 per cent of protein (casein). These rats grew nearly to the same length as control rats raised on a normal ration, but at all ages they were lighter in weight than the controls, apparently due to an almost total suppression of fat storage in the adipose tissues. Urine examination showed a larger excretion of protein per gram of renal tissue in the high-protein rats than in the controls, though at autopsy no pathological changes were found in the kidneys. An increased weight of kidney and a decreased weight of liver were also noted in the high-protein rats. The substitution of 1 per cent of cystine for the same amount of starch in the control diet produced no observable effect on the kidneys of a third group of rats thus fed.*

Perhaps the most convincing demonstration of the harmlessness of protein *per se,* even when consumed in great excess, is afforded by the published investigations of Osborne and Mendel and their associates upon rats. In 1921 [138] they reported preliminary results of feeding to rats rations that consisted entirely of protein (casein) with the exception of appropriate supplies of inorganic salts and of vitamine A and B. The animals readily tripled their initial weight (about 60 grams) on such diets. Later [139] they demonstrated that rats could grow to weights exceeding 200 grams at a good rate even on these extremes of diet that furnished only minimal amounts of energy in the form of fat or carbohydrate.† When the ration was less one-sided in composition, yet contained at least 50 per cent of protein (casein, edestin, or lean beef), excellent growth was attained.

Having observed a striking enlargement of the kidneys in rats that

[135] Kennedy, W. P., *Quart. J. Exper. Physiol.,* 1926, xvi, 281. *See Chem. Abst.,* 1927, xxi, 119.

[136] Miller, A. J., *J. Exper. Med.,* 1925, xlii, 897.

[137] Addis, T., MacKay, E. M., and MacKay, L. L., *J. Biol. Chem.,* 1926, lxxi, 139.

* Apparently through a curious oversight, the numbers of rats used in these experiments were not reported, nor are statistical measures of variability given.

[138] Osborne, T. B., and Mendel, L. B., *Proc. Soc. Exp. Biol. Med.,* 1921, xviii, 167.

[139] Osborne, T. B., and Mendel, L. B., *Proc. Nat. Acad. Sci.,* 1921, vii, 157.

† These rations contained 95 per cent of protein, consisting of 90 per cent of casein or meat residue, and 5 per cent of either zein or gliadin.

were kept for some time on diets rich in protein,[140] these high-protein feeding studies have been extended by Osborne, Mendel, Park and Winternitz[141] to bear specifically upon the kidney changes produced. They were able to confirm the earlier observations of large increments of body weight in rats at an essentially normal rate and to adult size on diets whose protein component represented two-thirds or more of their energy content. Also animals raised to large size on diets of a relatively low protein concentration could adjust themselves with no apparent detriment to these protein-rich dietaries.

The blood of animals subsisting on these diets was somewhat richer in non-protein and urea nitrogen than the blood of control rats, but urine examinations revealed no abnormalities indicative of chronic renal disorders attributable to the protein intake. Hypertrophy of the kidneys was found to be a characteristic feature of rats subsisting on a diet in which the protein contained one-third or more of the metabolizable energy. The period of time required to bring about these hypertrophies was surprisingly brief. After 8 days of high-protein feeding, unmistakable signs of kidney enlargement were noted. Histological examinations of these enlarged kidneys showed certain differences in structure as compared with the normal kidney, but nothing that the investigators could recognize as evidence of a nephritic condition was observed.*

MacKay, MacKay and Addis[142] have recently shown very clearly that kidney hypertrophy may be produced readily in young rats by high-protein feeding, but that in adult rats, the kidney responds but slightly to an increase in protein intake. That the hypertrophy induced in young rats was not due to the added work of eliminating greatly increased amounts of urea was shown by the negative effect on the kidney of additions of urea to a diet of moderate protein content.

Smith, Moise and Jones[143] have also reported the results of experiments on rats demonstrating the much greater adaptability of the young animal to sudden increases in the excretory work of the kidney, occasioned either by unilateral nephrectomy, the ingestion of high-protein diets, or both. However, the histological evidence of renal injury induced

[140] Osborne, T. B., Mendel L. B., Park, E. A., and Darrow, D., *Proc. Soc. Exp. Biol. Med.*, 1923, xx, 452. *See also,* Osborne and Mendel, *J. Biol. Chem.*, 1924, lix, 13.

[141] Osborne, T. B., Mendel, L. B., Park, E. A., and Winternitz, M. C., *J. Biol. Chem.*, 1927, lxxi, 317.

* Only extremely slight renal lesions were generally observed in rats that had consumed high protein synthetic diets for 200 to 364 days, and these lesions were similar to those observed in control rats of a more advanced age. However, 5 rats kept for over a year on the Polvogt, McCollum and Simmonds high-protein diet, made up of natural foods, all showed severe renal lesions.

[142] MacKay, L. L., MacKay E. M., and Addis, T., *Proc. Soc. Exp. Biol. Med.*, 1927, xxiv, 335, 336. *See also, Amer. J. Physiol.*, 1928, lxxxvi, 459, 466.

[143] Smith, A. H., Moise, T. S., and Jones, M. H., *Proc. Soc. Exp. Biol. Med.*, 1927, xxiv, 746. *See also,* Moise and Smith, *J. Exp. Med.*, 1927, xlvi, 27.

experimentally in adult rats was not of the nature of nephritis, but resembled the senescent focal lesions that are found in aged rats.

Since so many instances of successful nutrition over extended periods of high-protein feeding have been recorded in the literature, it may perhaps be considered as demonstrated that excessive protein intakes are not necessarily harmful, particularly if initiated in adolescence, and that moderately high protein intakes *per se* are quite incapable of producing organic injury. The exceptional reports of renal injury accompanying a high-protein dietary régime are probably explainable by references to other factors than protein, such as the specific effect of certain foods, while the evidences of renal disturbances noted by Squier and Newburgh in man following high-protein meals may reasonably be considered as temporary effects that may eventually disappear as the kidneys adapt themselves to the increased demands upon them.

The mass of experimental work just reviewed, demonstrating the compatibility of continued health and normal nutrition with protracted high-protein feeding, discount considerably the significance of observations apparently relating such dietaries with vascular disturbances. Using incomplete diets and no control groups of animals, Newburgh and Squier,[144] and later Newburgh and Clarkson,[145] have reported the production of a diseased condition of the aorta (arteriosclerosis, atherosclerosis) in rabbits by diets rich in protein, while Nuzum, Osborne, and Sansum,[146] in experiments that meet the objections raised against the former work, observed the appearance and persistence of hypertension and renal irritation in rabbits on moderately high protein rations. They hesitate, however, to ascribe these results to the protein *per se,* suggesting that diets containing excessive acid or alkaline ash might in themselves be responsible for degenerative blood vessel and kidney changes. However, Addis, MacKay and MacKay[147] have adduced evidence against such an explanation.

Concerning the work of Nuzum, Osborne and Sansum, it may be said that, if Chart 1 of their paper represents the changes in blood pressure from the beginning of the periods of experimental feeding, only the rabbits on the liver diet of McCollum show any progressive increase in arterial tension. The soybean diet, containing only slightly less protein, actually produced a decrease in blood pressure if this interpretation of the chart is correct. The final blood pressures observed at the end of one

[144] Newburgh, L. H., and Squier, T. L., *Arch. Intern. Med.,* 1920, xxvi, 38.
[145] Newburgh L. H., and Clarkson, S., *J. Amer. Med. Assoc.,* 1922, lxxix, 1106.
[146] Nuzum, F. R., Osborne, M., and Sansum, W. D., *Arch. Intern. Med.,* 1925, xxxv, 492. In a later article (*Arch. Intern. Med.,* 1927, xl, 364) Nuzum reports other investigations of like significance.
[147] Addis, T., MacKay, E. M., and MacKay, L. L., *J. Biol. Chem.,* 1926, lxxi, 157.

year on the experimental diets were not in proportion to the protein contents of the diets nor to the recorded contents of the blood in non-protein and urea nitrogen. Throughout the experiment only the control rabbits showed any considerable growth, indicating the possibility that the test diets were, for some reason, unsatisfactory for this type of animal.

It may be mentioned that Strouse and Kelman [148] have not noticed any relation between protein feeding and blood pressure in observations upon patients with hypertension, while other observations of a similar nature may also be found in the older literature.[149] Any adequate review of the clinical phase of the subject cannot be attempted here.

The investigations of Major and his coworkers [150] relating arterial hypertension to the production of guanidine compounds in metabolism is interesting, but the definite demonstration of such a relation is still waiting upon adequate methods for the quantitative analysis of body fluids and secretions for these compounds. The harmful effects that Hartwell [151] has noted in the young of mother rats fed upon excess protein are difficult to interpret, particularly since the rations used may not have been adequate in other respects. That excess protein *per se* does not impair successful reproduction and lactation appears clearly established from the experiments of Polvogt, McCollum, and Simmonds.[130]

The experimental feeding of human subjects over considerable periods of time on high protein diets has not thus far been attempted, insofar as the writers are aware. However, several reliable observations upon the Eskimos, who are essentially a carnivorous people, can serve the same purpose. A. and M. Krogh [152] in 1913 reported on the dietetic habits of the Eskimos, showing that a daily consumption of 500 grams of protein and over was not unusual and was not associated with ill-health. Lieb [153] observed that Eskimos living exclusively upon meat for long periods of time were in good health, free from constipation, and finally Thomas [154] has reported a comprehensive cardiac, vascular, and renal survey of the Eskimos of northern Labrador and of Greenland, made in 1926 by personal observation and by study of the medical records and personal experiences of Danish physicians residing in Greenland. Quoting Thomas' report:

[148] Strouse, S., and Kelman, S. R., *Arch. Intern. Med.*, 1923, xxxi, 151.
[149] Linossier, G., *Med. Klin.*, 1913, ix, 2143. *See also,* Mosenthal, H. O., *Am. J. Med. Sci.*, 1920, clx, 808.
[150] Major, R. H., and Stephenson, W., *Bull. Johns Hopkins Hosp.*, 1924, xxxv, 140, 186; Major, *ibid.*, 1925, xxxvi, 357; Major and Weber, C. J., *ibid.*, 1927, xl, 85; Major, *J. Am. Med. Assoc.*, 1924, lxxxiii, 81.
[151] Hartwell, G. A., *Lancet*, 1921, I, 1240; *Biochem. J.*, 1922, xvi, 78; *ibid.*, 1924, xviii, 785.
[152] Krogh A., and Krogh, M., "Meddelelser om Groonland," 1913, 51; *Abst. Chem. Soc.*, 1914, i, 106.
[153] Lieb, C. W., *J. Amer. Med. Assoc.*, 1926, lxxxvii, 25.
[154] Thomas, W. A., *J. Amer. Med. Assoc.*, 1927, lxxxviii, 1559.

"Nearly all of the Greenland Eskimos have no access to vegetable food and have, since being weaned, lived on an exclusively carnivorous diet, usually eaten raw. Very little fat or blubber is eaten; the red meat and liver are preferred, but with inadequate supply all parts of the animal are eaten. The observations show conclusively that there is no unusual prevalence of vascular or renal disease; scurvy and rickets did not appear among these Greenland Eskimos. The Labrador Eskimo, however, whose meat is cooked and whose diet includes many prepared, dried and canned articles, is very subject to both these deficiency diseases."

A comparison of Hindhede's extensive experiences with low-protein diets [122] with these recorded observations upon the carnivorous Eskimos justifies the conclusion that good health and successful nutrition are compatible with a wide range of protein intake. Protein does not appear to be inherently harmful as many presume it to be.

While it is probably true that protein may be indulged in to great excess without any immediate ill effects, or even with no ill effects at all, the wisdom of so doing may be questioned. The comfort of an individual, as well as his mental and physical efficiency are probably adversely affected under certain conditions by a high-protein dietary. The well-known stimulating action of protein foods on metabolism may be related to vitality and stamina; at the same time the pronounced heating effect of protein associated with its stimulating action will naturally and rightly lead to a seasonal variation in the popularity of protein foods.

PELLAGRA

It has been claimed that pellagra is a disease due to amino-acid deficiencies in the same way that beri-beri, scurvy and rickets are due to vitamin deficiencies. Since pellagra, or a disease of similar etiology, has not certainly * been induced in experimental animals, arguments concerning the cause of pellagra have largely been based upon statistical observations of the diet of pellagrins as compared with that of non-pellagrins under the same hygienic and other environmental conditions. More recently Goldberger and his associates of the United States Public Health Service have employed successfully the experimental method at the Georgia State Sanitarium. It is particularly with these experimental studies that the following discussion is concerned.

* The most recent investigations of Goldberger and his associates in the U. S. Public Health Service are rendering more and more probable the etiological identity of black-tongue in dogs with pellagra in humans. The former disease was first induced experimentally in dogs by Chittenden and Underhill (*Amer. J. Physiol.*, 1917, xliv, 13). A recent note by Miss Kunde (*Proc. Soc. Exp. Biol. Med.*, 1926, xxiii, 812) on the development of pellagra-like skin lesions in rabbits 8 to 20 months after complete thyroidectomy is of interest in this connection.

These statistical and experimental observations have established clearly that dietetic error or errors of some description are primarily responsible for the onset of pellagra, and that the presence in the diet of certain amounts of protein-rich animal foods, particularly milk and meat, is an effective preventative against this disease.[155]

Goldberger and Tanner [156] have shown that neither mineral supplements to a pellagrous diet nor additions of standard sources of the known vitamins, exert a protective action against pellagra. They were led by these observations to the conclusion that "the dominating rôle of diet in the prevention and causation of pellagra must be referred primarily to the character of the protein (amino acid) supply." This conclusion apparently does not take sufficient cognizance of the fact that we have no assurance that all of the dietary factors essential to the health and proper nutrition of the human species are known.

The later work of these investigators is leading them farther and farther away from this earlier belief. In 1925, Goldberger and Tanner [157] reported that dried legumes rich in protein (soybeans and cowpeas) were relatively ineffective in protecting against pellagra, and that even purified casein (85 to 90 grams per day) was only partially successful. It appeared evident to them that "a liberal supply of protein presumably of good biological quality does not completely prevent, though it may modify, the clinical picture of pellagra by notably delaying or preventing the development of the distinctive dermatitis," and that "in the prevention (and, presumably, causation) of pellagra there is concerned a hitherto unrecognized or unappreciated dietary factor which we designate as factor P-P" which "may possibly play the sole essential rôle in the prevention (and causation) of pellagra." Brewer's yeast, fresh milk and fresh lean beef appear to be good sources of factor P-P. Further evidence of the same character was reported in 1926 by Goldberger, Wheeler, Lillie, and Rogers; [158] in particular the exceptional effectiveness of dried yeast and dried yeast extracts in amounts as low as 15 grams per day was demonstrated.

The rôle of protein and amino acids is thus minimized if not removed from consideration entirely, and the existence of another vitamin essential to health seems rather clearly indicated. The possibility that factor P-P is identical with the growth-promoting essential commonly included with the antineuritic vitamin in the term "vitamin B" is dis-

[155] Goldberger, J., Wheeler, G. A., and Sydenstricker, E., *U. S. Public Health Rpts.*, 1920, xxxv. 648.
[156] Goldberger, J., and Tanner, W. F., *U. S. Public Health Rpts.*, 1922, xxxvii, 462.
[157] Goldberger, J., and Tanner, W. F., *U. S. Public Health Rpts.*, 1925, xl, 54. 1926, xli, 297.
[158] Goldberger, J., Wheeler, G. A., Lillie, R. D., and Rogers, L. M., *U. S. Public Health Rpts.*, 1926, xli, 297.

cussed in the latter paper in the light of preliminary experiments with rats.

ADEQUATE AND OPTIMUM NUTRITION

The question of the proper amount of protein to include in the diet is intimately involved in the conception of optimum nutrition. By "optimum" nutrition is meant a condition of animal functioning induced by liberal feeding that is superior to merely "adequate" or "normal" nutrition. It implies that a supply of nutrient material possessing specific functions in the body above the requirements for those functions will in some manner induce supernormal functioning, which in turn appears to imply that the mere presence of an excess of nutriment in the body fluids exerts a favorable physiological effect.

Evidently the postulate of an optimum nutrition superior to merely adequate nutrition cannot be substantiated until a most searching and successful study of nutritive requirements has been completed, in order to prove that the condition of adequate nutrition with which the optimum is to be compared is in truth adequate. In such studies it must be realized that there are for each required nutrient and each animal function probably two types of requirements and hence two conceptions of adequate nutrition. One relates to the requirement of that nutrient for the successful performance of the function in question. Thus, a certain assortment of amino acids and a certain intake of each are essential to the maintenance of life, to growth, to the production of young, and to their successful nourishment during the period of their dependence upon the mother. If this requirement is not satisfied by the diet a failure of functioning occurs and continued existence of the species, if not of the individual, is impossible. In the case of the vitamins, inadequate nutrition in this sense is manifested by the occurrence of deficiency diseases.

But the satisfaction of these requirements cannot be considered as the attainment of adequate nutrition, except in a very restricted sense. Many animal functions, if not all, can be performed in different degrees. A recent contribution of Osborne and Mendel [159] illustrates the extremes of growth that may be induced by different diets in animals of the same genetic constitution, in so far as growth may be measured by mere increase in weight. Fertility and lactation are also variable functions. If the term "adequate" nutrition has any definite meaning at all, when applied to these functions, it would seem to refer to a nutritive régime sufficiently liberal to provide for a maximal performance. Any nutritive

[159] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1926, lxix, 661.

régime less liberal than this could be called adequate only on purely arbitrary grounds.

It may seem that the whole question at issue here is merely one of the definition of terms, in particular the substitution of the word "adequate" to cover what is frequently referred to as "optimal." This substitution, however, replaces an indefinite term, appearing to involve implications difficult of acceptance, by a term perfectly definite in its significance. Adequate nutrition, in the sense proposed in the preceding paragraph, is very definitely related to an animal's food requirements as determined by its inherent functional capacities. Optimal nutrition has no such definite meaning and is sometimes understood to involve supernormal functioning.

The fact that a ration which is capable of nourishing rats successfully, generation after generation, can be altered so as to induce more rapid growth, earlier maturity, greater fertility, etc., has been considered of great significance,[160] and has been made the basis for speculations concerning the possibility of greatly improving human efficiency by similar alterations in diet, if not of producing supernormal men and women.[161] The former ration was called adequate, even though it did not, for example, promote one-half of the fertility of which the rat is capable; the latter ration, therefore, must be considered more than adequate, *i.e.,* optimal. The prevailing dietary habits in this country, for no obvious reason at all, are considered merely adequate; hence the possibility of increased human efficiency and happiness following super-adequate nutrition.

The great significance attached by Sherman to the marked improvements in the nutrition of rats induced by increasing the proportion of milk in the diet, is greatly diminished when it is realized that the inferior ration, containing the smaller proportion of milk solids, cannot fairly be called nutritively adequate, except insofar as it is capable of promoting *continuously* the different functions of animal life. However, the extent to which these functions operate should also be considered in assessing the adequacy of any dietetic régime: *i.e.,* adequate nutrition implies something more than continued health and existence. The experiments of Sherman simply afford an illustration of the different degrees of adequacy of different rations, and not of a super-adequate nutrition following upon increases in the consumption of milk.

In this connection it may be questioned whether any dietary régime is ideal that is maintained constant throughout life. Feeding experiments

[160] Sherman, H. C., and Campbell, H. L., *J. Biol. Chem.,* 1924, lx, 5.
[161] Sherman, H. C., *J. Ind. Eng. Chem.,* 1926, xviii, 1261.

on rats concerned with so-called optimal nutrition, appear to be based upon the assumption that the food requirements of the animal under the greatest nutritive strain, for example when nursing a large litter of young, should determine the composition of the diet to be given at all times. This is equivalent to assuming that the dairy herd should be maintained constantly on a ration capable of supporting the milk production of the largest-producing individual females; that the ration of a poultry flock should be determined by the nutritive requirements of the most productive hens; and that a human community should be nourished by a ration adapted to the needs of the child at the period of most rapid growth, or, if these are more intense, to the needs of the nursing mother. Nutritive requirements vary for different individuals and for different physiological functions and should therefore be studied with reference to particular conditions. It is doubtful whether the differences between the nutritive values of rations can be intelligently studied by feeding them continuously to experimental animals through several generations. It would seem that they could be more precisely compared by reference separately to each distinct type of physiological function.

SUBSTITUTES FOR PROTEIN

The question of the value of non-protein nitrogenous substances as substitutes for protein in the feeding of animals has occupied the attention of a great number of investigators, and is the subject of many experimental reports in the literature, dating back into the middle of the last century. The problem has been approached from the purely scientific standpoint of investigating the synthetic powers of the animal organism, as well as from the practical standpoint of discovering cheap substitutes for protein in the feeding of farm animals.

The task of reviewing all of this work and of attempting to assess the significance of each contribution in an endeavor to arrive at conclusions compatible with the greatest number, is on the face of it so prodigious as to discourage its undertaking here. Bibliographies of these investigations and discussions of them have been published by Cathcart [162] in 1920, and by Scharrer and Strobel [163] in 1925. Investigations of this nature, particularly from German agricultural experiment stations, have been reviewed by Armsby [164] up to 1911. Therefore, only the work of the last few years will be considered in any detail. Its interest and importance, particularly from the agricultural standpoint, warrants a somewhat extended discussion of it.

[162] Cathcart, E. P., "The Physiology of Protein Metabolism." London, 1921, pp. 44 to 50 *See also* Albertoni, P., *Ergebn. Physiol.*, 1921, xix, 594.
[163] Scharrer, K., and Strobel, A., *Z. angew. Chem.*, 1925, xxxviii, 601.
[164] Armsby, H. P., *U. S. Dept. Agr., Bur. An. Ind. Bull.* 139, 1911.

It requires no experimentation to establish the fact that the non-protein nitrogenous substances of food materials may contribute to their protein value in nutrition insofar as they consist of amino acids or their derivatives. For this reason the crude protein content of foods is a better indication of their value as sources of body protein and the non-protein substances of the tissues than is the true protein content. The question at issue relates to the value of individual nitrogenous compounds of low molecular weight, such as ammonium salts, asparagine, and urea, in serving the peculiar functions of proteins in the body when added to the ration of an animal in massive doses.

Experiments on carnivorous and omnivorous animals have not given any clear indication of a utilization of ammonium salts and urea in the anabolic processes of the body. It is true that an apparent retention of the nitrogen of ammonium salts, particularly of the organic acids, has been frequently noted, but the significance of such estimated retentions is considerably disturbed by the fact, shown especially by Pescheck [165] and by Abderhalden,[166] that sodium and magnesium salts of organic acids, as well as sodium nitrate, could favorably affect the nitrogen balances of animals and thus present the same *appearance* of protein sparing as ammonium salts themselves.

Thus, temporary fluctuations in the estimated nitrogen balance of an animal induced by the ingestion of large amounts of simple nitrogenous compounds, or by the disturbances in the acid-base equilibrium of the tissues, are not necessarily interpretable in terms of protein storage or protein catabolism. Gessler [167] has shown that the retention of ammonium nitrogen by dogs is not associated with a retention of sulfur and hence can hardly be indicative of protein storage, while the experiments of Adolph [168] illustrate the importance of the acid-base condition of the body in determining the rate of elimination of ingested ammonium salts and urea.

Grafe's work in particular has been interpreted as demonstrating the ability of the animal body to synthesize protein from ammonium salts. However, his nitrogen metabolism data throughout are so variable as to indicate that experimental conditions have not been sufficiently under control; the interpretation of nitrogen balances under these conditions is difficult and the criticism of Underhill and Goldschmidt [169] of Grafe's procedures seems well founded. Surely the contention of Grafe [170] that

[165] Pescheck, E., *Arch. ges. Physiol.*, 1911, cxlii, 143; *Biochem. Z.*, 1912, xlv, 244; *ibid.*, 1913, lii, 275; *ibid.*, 1914, lxii, 186.
[166] Abderhalden, E. *Z., physiol. Chem.*, 1916, xcvi, 1.
[167] Gessler, H., *Z. physiol. Chem.*, 1920, cix, 280.
[168] Adolph, E. F., *Amer. J. Physiol.*, 1924-25, lxxi, 355.
[169] Underhill. F. P., and Goldschmidt, S., *J. Biol. Chem.*, 1913, xv, 341.
[170] Grafe, E., *Z. physiol. Chem.*, 1912, lxxviii, 485.

a dog may be maintained in nitrogen equilibrium for considerable periods of time on ammonium salts added to an abundant carbohydrate diet cannot be accepted. The indispensability of tryptophane, cystine and histidine for the maintenance of life has been too firmly established to be impaired by the variable experimental data that Grafe presents. Furthermore, it is hardly conceivable that ammonium salts are more effective than incomplete proteins, such as gelatin and zein, in maintaining the nitrogenous integrity of the animal organism or in promoting protein synthesis. The inability of these proteins in covering both of these functions has been abundantly demonstrated.

It seems fair to conclude, therefore, that, although apparent and temporary retentions of nitrogen have been observed after the feeding of ammonium salts to animals, their significance is still obscure. It seems premature to conclude that such observed retentions are indicative either of protein synthesis or even of protein sparing. The assumption that the feeding of ammonium salts will retard or even reverse the deamination reactions to which amino acids are subjected as the initial step in their catabolism, is also premature until it is shown that such feeding will appreciably alter the concentration of ammonia in the tissues in which these reactions are occurring. In this connection, the small and constant concentration of ammonia in the systemic blood, in spite of a large and variable influx from the intestinal tract, seems significant. Also, the mass action theory of the effect of ammonium salt feeding seems inconsistent with the variable nitrogen retentions obtained with different ammonium salts, particularly the greater effect of organic as compared with inorganic salts.

The same general conclusion in regard to the feeding of urea and individual amino acids and amino acid amides seems justified, *i.e.*, that no incontrovertible evidence has thus far been secured in regard to their utilization in anabolism.

While the utilization of individual non-protein nitrogenous substances as protein substitutes by non-ruminants is at least doubtful, much more significant results have been obtained with ruminants, as Armsby [164] has clearly shown. Zuntz appears to have been the first to suggest that the difference in the results obtained with the two classes of animals may be due to the difference in their digestive processes. The ruminant is provided with a voluminous paunch in which much of the food consumed is subjected to a vigorous action of various forms of microörganisms, evidenced by the production and excretion from the alimentary tract of considerable amounts of methane gas. It is conceivable that ingested non-protein nitrogenous compounds influ-

ence the protein metabolism of the animal indirectly in a number of ways:

1. These non-protein substances may be more readily available as sources of nitrogen to the paunch microörganisms (bacteria, protozoa, and infusoria [171]) than the food protein, and hence their ingestion may stimulate the fermentation of cellulose. The extra organic acids thus produced may, after absorption, exert a protein-sparing effect, and hence favor increased nitrogen retention.

2. The non-proteins may spare food protein in the paunch from decomposition by the flora implanted there, and thus favor increased nitrogen retention.

3. The non-proteins may be built up into the cellular proteins of the microörganisms, which later are digested in the true stomach. Thus, an addition to the protein supply would result, with increased opportunities for nitrogen retention.

The latter explanation is the one most commonly advocated by investigators in this field. According to this explanation the paunch of the ruminant is endowed with another important function. Besides affording a site for the fermentation of the celluloses and hemicelluloses of the feed that otherwise would pass through the alimentary canal unscathed, the paunch is looked upon as a protein factory, in which soluble non-protein nitrogenous substances of various kinds are built up into protein, which is later hydrolyzed to its constituent amino acids in the abomasum and the small intestine, and absorbed.

THE VALUE OF UREA AS A PROTEIN SUBSTITUTE

During the World War and after, the scarcity of nitrogenous concentrate feeds for live stock in Germany stimulated a large amount of investigation of the possibility suggested by the earlier work of using non-protein nitrogenous substances in covering, in part at least, the protein requirements of farm animals for maintenance, growth and milk production. The almost simultaneous development of a process by which urea can be manufactured cheaply from atmospheric nitrogen and carbon dioxide naturally led to the use of this compound largely in such investigations.

The first investigator to take up the problem of the value of urea as a substitute for protein in the feeding of ruminants was Völtz of the Agricultural College at Berlin. In an extensive series of metabolism experiments on 3 Merino lambs about 9 months old, Völtz obtained [172]

[171] Ellenberger, W., and Scheunert, A. S., "Lehrbuch der vergleichenden Physiologie der Haussäugetiere," 1910, p. 357; Liebe.anz, E., *Arch. Protistenkunde*, 1910, xix, 19; Henneberg, W., *Berl. klin. Wochn.*, 1919, lvi, 693; Eberlein, R., *Z. wiss. Zool.*, 1895, lix, 233.

[172] Völtz, W., *Biochem. Z.*, 1920, cii, 151.

remarkable results with rations extremely low in nitrogen to which 30 grams of urea per head per day were added. The daily basal ration consisted at the start of 500 grams of rye straw or chaff, digested for 18 hours with dilute alkali and washed according to the Beckmann process, 100 grams of potato starch, 100 grams of sugar and 19.2 grams of a salt mixture. In later periods the daily allowance of salts was about doubled, the treatment of the rye straw (or chaff) was modified somewhat, and the intake of food was varied in accordance with the appetites of the animals. The rye straw and rye chaff contained only from 2 to 4 per cent of crude protein, while the total nitrogen of the basal ration was not equal to the metabolic nitrogen of the feces produced from it.

While the experimental data on two of the three lambs were not particularly impressive, the third lamb was fed continuously on this abnormal ration for a period of 242 days, during which period the body weight increased from 29.2 kgms. to 41.2 kgms. For all but the last 87 days, the urine and feces were collected continuously and the nitrogen balances calculated for seven successive sub-periods. The average nitrogen balance in each period was positive. In this experiment it seems impossible to escape the conviction that urea has successfully replaced protein for maintenance and probably for growth also. The experiment is noteworthy also for the fact that no source of vitamins was added to the ration, except the digested and washed straw or chaff, which could hardly have furnished any considerable amounts of these dietary factors.

The value of any practicable and feasible method of substituting cheap non-protein substances for protein concentrate feeds relates particularly to the feeding of milk cows, since with these animals protein concentrates are commonly used in order to secure the maximum production. In a second report, dated two years after his first, Völtz, in coöperation with Dietrich and Jantzon,[173] published the results of a number of feeding experiments on dairy cows involving urea additions to low protein rations, and the substitution of peanut meal in dairy rations by urea and some starchy food such as potatoes. In addition to these simple feeding experiments, nitrogen metabolism studies were made on one sheep and one lactating cow on urea-containing rations and urea-free rations.

Considering the latter experiments first, the one sheep was fed a ration of hay, steamed and fermented potatoes, and salts for a period of 22 days, the last 6 of which constituted a collection period; and the

[173] Völtz, W., Dietrich, W., and Jantzon, J., *Biochem. Z.*, 1922, cxxx, 323.

same ration with the addition of 15 grams of urea daily for the following 19 days, the last 10 of which involved the determination of the nitrogen balance. In the first period, the average daily balance was + 0.33 gram of nitrogen, and in the second period, + 1.37 grams. The body weight of the sheep remained approximately constant, and the digestion coefficients were not appreciably affected by the urea feeding. The large increase in the excretion of urinary nitrogen in the urea feeding period is consistent with the belief that most of the urea fed was simply absorbed and excreted, though evidently, unless urea was being excreted by other channels, some was being retained. Whether the urea nitrogen thus apparently retained was serving, directly or indirectly, as a protein substitute, is not very definitely indicated. The experiment would be more conclusive if a third experimental period, in which the sheep was again put upon the basal ration, had been undertaken.

A mature cow, producing from 3.06 to 4.85 kgms. of milk daily, was also made the subject of a metabolism study, consisting of 4 experimental periods. The basal ration of hay, sugar beets and minerals was fed in approximately constant amounts in all periods. In the first period this ration was fed alone; in the second period it was supplemented with peanut meal; in the third period it was supplemented with urea (105 grams) and potato flakes to equal the peanut meal supplement in nitrogen and energy value; and in the fourth period it was supplemented with urea only. The average daily milk production in the four periods was, respectively, 4.85 kgms., 4.82 kgms., 3.76 kgms., and 3.06 kgms., while the average daily nitrogen balances were, in order, — 15.66 grams, — 6.80 grams, — 3.88 grams, and — 5.91 grams. The variable amounts of nitrogen excreted in the milk (20.02, 20.13, 18.29, and 13.72 grams daily, respectively) render difficult the interpretation of the nitrogen balances. Evidently the potato flakes included in the ration in the third period exerted a more favorable effect on the nitrogen utilization, as revealed by a comparison of the data of the third and fourth periods, than the urea included in the ration of the fourth period, as revealed by a comparison of the data of the first and fourth periods. The precise value of urea in this test as a protein supplement is somewhat difficult to assess.

The feeding experiments of Völtz, Dietrich, and Jantzon, involving 4 other cows in milk, were planned in much the same fashion as the metabolism experiment just considered. The observations secured related to milk yields and changes in body weight and may be taken to indicate a favorable effect of the urea additions (150 to 178 grams

daily) on milk production. This effect is interpreted by the authors as the result of the indirect use of urea as a source of milk protein, through the mediation of the microörganisms of the paunch. From a comparison of the protein intakes in the different experimental feeding periods with the protein requirements of the cows as estimated from the feeding standards of Kellner, it is concluded that urea must have served in this way. However, the protein requirements for maintenance and milk production cannot be considered as having been accurately determined or as being accurately expressed in the Kellner standards. In fact, these standards, and most other feeding standards in current use, are probably excessive in their demands for protein.[174] They are to be considered more as safe guides in feeding practice rather than as accurate expressions of physiological requirements.

Arguments involving the use of these standards, therefore, are not particularly convincing, and in these experiments it appears within the realm of the possible that the protein required for both maintenance and milk production was furnished by the basal ration alone. The short feeding periods of three weeks or shorter, including the transitional feeding, are open to question, and the significance of the milk production records of only 6 to 12 days is uncertain. Inspection of the daily records leads to the suspicion that the observed effects of urea feeding may really have been only temporary and therefore of little practical importance. They are the result possibly of a direct stimulation of the mammary glands by the massive doses of urea given.

Although a number of other feeding and metabolism experiments on dairy cows concerned with the value of urea as a protein substitute have been reported from German institutions the results have been varied, and in no case, it would seem, has a demonstrably positive value been obtained, although in several cases the results were highly suggestive.

The experiments of Honcamp, Koudela, and Müller[175] are of this nature. One cow on a low protein ration of meadow hay, oat straw, dried beet pulp, and minerals did not respond to an addition of 90 grams of urea either in an increased milk production, in an increased digestion of her feed, or in a more favorable nitrogen balance. In this case the urea exerted an unfavorable rather than a favorable effect on the amount and composition of the milk and on the nitrogen balance.

In experiments on two other cows receiving a basal ration also low in protein, but higher in easily digestible carbohydrates (starches and

[174] Buschmann A., *Landw. Vers.-Stats.*, 1923, ci, 1.
[175] Honcamp, F., Koudela, S., and Müller, E., *Biochem. Z.*, 1923, cxliii, 111; Honcamp, F. *Z. angew. Chem.*, 1923, xxxvi, 45.

sugars), the addition of 150 grams of urea increased the milk production and appear to have exerted a markedly favorable effect on the nitrogen balance. However, the digestibility of all of the nutrients in the ration was distinctly higher in the periods of urea feeding than in the control periods. The digestibility of the total organic matter was higher by 10.9 per cent in the urea periods, that of the nitrogen-free extract, 9.3 and 8.5 per cent higher, and that of the crude fiber 26.4 and 30.7 per cent higher. While it appears probable that the urea might have been utilized indirectly in the protein metabolism of these cows, the increased amounts of digestible carbohydrates available to the animals in the urea periods undoubtedly exerted a considerable, though incommensurable, protein-sparing effect. A third cow, receiving practically the same treatment as the latter two, showed somewhat less favorable effects, while with two other cows on rations of normal protein content, the replacement of 30 to 40 per cent of the protein by urea exerted a slightly unfavorable effect on the milk production of one cow and a distinctly unfavorable effect on that of the other. The nitrogen balances of these two cows (positive in all periods) were also adversely affected, particularly in the case of the cow whose production of milk was also lowered.

The feeding periods in these experiments of Honcamp, Koudela, and Müller were of only 16 days' duration and the periods for which complete data were secured were of only 8 days' duration. Temporary effects of urea on the mammary function, on digestibility, and particularly on the nitrogen balance are emphasized in such an experimental procedure.

In a later publication,[176] the same investigators showed, also by a series of short feeding periods (10 days transitional and 10 days experimental) on 9 cows, that the replacement of 26 per cent of the protein in a dairy ration containing amounts of this nutrient adequate according to the Kellner standards, by urea and dried beet pulp of equivalent nitrogen and starch value, had no distinct effect on the milk production of the cows, though the body weight decreased slightly. However, in the urea periods, the ration contained enough protein according to the Fingerling standard for the maintenance of the cows and for the milk production secured. In a second experiment involving 20 cows, and feeding periods somewhat shorter than in the first experiment (18 days, of which the last 8 days only are considered), the addition of 150 grams of urea per head per day to either a low protein or a moderate protein ration, induced slight but quite general increases

[176] Honcamp, F., Koudela, S., and Müller, E., *Landw. Vers.-Stats.*, 1924, cii, 311.

in the production of milk and butterfat (generally less than 10 per cent). Losses in body weight by many of the cows complicates the interpretation of these results.

A series of feeding experiments on 33 cows, in which the value of urea as a protein substitute was investigated, was reported by Hansen [177] from the University of Königsberg. In the first of these experiments, in which 6 cows were used in 3 feeding periods of 18 days each, neither the addition of urea (200 grams per head daily) to a ration of hay and dried sugar beet pulp, nor its substitution for soybean protein in a similar ration including some of this concentrate, produced any appreciable effect on milk production. In the third experiment on 4 cows in feeding periods of 21 days, the substitution of urea and dried beet pulp for protein concentrates in a normal dairy ration depressed the milk production slightly and occasioned general decreases in weight. Although the protein intake in the urea periods was undoubtedly low and quite probably inadequate, the losses in weight of the cows in these periods precludes the conclusion that urea was serving the place of feed proteins.

The fourth of Hansen's experiments was planned very much like the second and the results bear approximately the same significance. The fifth experiment differed from the others in the length of the period of urea feeding, *i.e.*, 91 days; the substitution of urea (100 to 200 grams daily) and potato flakes for nitrogenous meals appeared to have depressed somewhat the normal course of milk production, but the weights of the cows were well maintained. The evidence presented to prove that the protein requirements of the cows in the urea period could not have been supplied by the crude protein of the basal ration is not complete, since the estimates of the protein required for both maintenance and milk production may well be excessive, particularly the estimate for maintenance. The experiment, therefore, shows simply that the continued feeding of these massive doses of urea is without harmful effect; the results in the urea period are perhaps more readily explainable on the basis of a bacterial synthesis of protein from urea, but they have not been shown to be incompatible with alternative explanations.

The sixth experiment of Hansen was again concerned with the substitution, in short feeding periods (20 days), of urea plus potato flakes for nitrogenous concentrates. With 9 cows, the amount of milk fell off in each case in the urea period, although the milk fat produced increased slightly. However, again no successful demonstration that

[177] Hansen, J., *Landw. Jahrb.*, 1922, lvii, 141.

the crude protein in the basal ration of the urea period could not have covered the nitrogenous requirements of the cows is given. Hence, the experiment proves nothing except that the protein contents of the rations of the control periods were excessive. In the last experiment, the addition of urea to low-protein rations containing considerable potato flakes increased but slightly the milk production of six cows, an increase that may have been caused by an increased digestibility of the carbonaceous feeds. One may wonder why in this experiment, in which conditions appeared most favorable for a bacterial synthesis of protein from urea, greater effects of urea additions were not obtained.

The experiments of Hansen are similar in their import to a number of other experiments on dairy cows or lactating goats, among which may be mentioned those of Paasch,[178] Ungerer,[179] Richardsen and Brinkmann,[180] and Morgen, Windheuser and Ohlmer.[181] From all of these experiments, it appears that the value of urea, and other single non-protein nitrogenous substances when fed in massive doses, as a source of milk protein, is yet to be demonstrated. The problem is a difficult one to attack in a direct and effective manner, but it would seem that the experimental technic exemplified in this work could be improved upon. The necessity of making numerous and involved calculations based upon doubtful estimations of protein requirements in order to arrive at conclusions of the value of urea is sufficient indication that the experimental procedure is either at fault, or is inadequate to serve the purpose desired until certain necessary information is at hand.

Much of the experimental work on the feeding of urea indicates no favorable effect on protein utilization. Scheunert, Klein and Steuber [182] have reported a very careful metabolism study on 2 growing lambs and 3 full-grown wethers that throw a different light upon some of the investigations previously reported. The 2-year-old wethers were fed a basal ration extremely low in nitrogen, consisting of straw treated by the Lingner process (3.0 per cent of crude protein), potato meal (4.31 per cent crude protein), and a mixture of salts. With the addition of 20 grams of urea per day to this ration, one wether was fed for 69 days and in 3 balance periods was found to be storing nitrogen at an average rate of 2.3 grams per day; a second wether was fed for 46 days and in 2 balance periods was found to have an average daily nitrogen balance of 2.7 grams; while the third wether was fed for 57 days and in one balance period at the end of this period was found to

[178] Paasch, E., *Biochem. Z.*, 1925, clx, 333; *Landw. Jahr.*, 1926, lxiv, 495.
[179] Ungerer, E., *Biochem. Z.*, 1924, cxlvii, 275.
[180] Richardsen and Brinkmann, *Fühlings Landw. Ztg.*, 1922, lxxi, 325; *Exp. Sta. Rec.*, 1923, xlviii, 670.
[181] Morgen, A., Windheuser, C., and Ohlmer, E., *Landw. Vers.-Stats.*, 1922, xcix, 359.
[182] Scheunert, A., Klein, W., and Steuber, M., *Biochem. Z.*, 1922, cxxxiii, 137.

be in positive nitrogen balance. After initial losses in weight, all three wethers maintained their weights approximately constant. In the case of the first and third wethers, the nitrogen balances were determined for several days after the withdrawal of urea from the ration and were found to be distinctly negative. The digestibility of carbohydrates was also depressed markedly in the final non-urea period for one wether.

The results of these experiments at their face value support fully the belief of Völtz that urea can, under such conditions of feeding, serve the function of protein in the animal body. But Scheunert and his associates were led to doubt this obvious interpretation on the basis of the results of respiration experiments on one of the wethers. These experiments indicated more or less certainly that the sheep was storing material in its body which, if oxidized, would produce considerably more CO_2 and consume considerably less oxygen than would the amount of stored protein estimated according to the nitrogen metabolism data. It was suspected, therefore, that the urea consumed must have been excreted by other channels than the kidneys. An examination of a small portion of wool from this sheep showed that it contained rather large amounts of extractable nitrogen.

In order to confirm, if possible, the belief that sheep receiving large doses of urea excrete some of it through the skin, thus vitiating the significance of the nitrogen balance as commonly computed, another experiment was undertaken with a year-old lamb, which was fed a very low-protein ration similar to that previously used plus a daily dose of 20 grams of urea. On this feeding a positive nitrogen balance was soon established and the characteristic effect of urea in improving the digestibility of the non-nitrogenous nutrients of the ration was soon observed. After 24 days of urea feeding the animal was thoroughly washed with soap and water, and again 8 days later. The latter washing contained about 8 grams of nitrogen, indicating an excretion through the skin of approximately 1 gram of nitrogen daily, an amount very nearly equal to the computed nitrogen balance.

After 88 days of urea feeding, the basal ration of straw, potato meal, starch, oil and salts was fed alone. The nitrogen in the urine, after a single day, reached the low level of less than a gram per day, suggesting a rapid flushing out of circulating urea, rather than a gradual catabolism of "deposit protein." In the absence of urea, reductions had to be made in the ration, but after 2 weeks a slight positive nitrogen balance was established. The digestion of starch became constantly poorer and the appetite of the animal decreased, so that after 3 weeks of feeding without urea this type of feeding had to be terminated.

In order to obtain information as to the excretion of nitrogen through the skin during a dietary régime without urea, the animal was thoroughly washed the day before and the day after the termination of experimental feeding. Only a trace of nitrogen was found in these washings. Since this condition may have been due to the low protein feeding, the animal was put upon a natural ration of oats, hay, starch, gluten, and oil, containing in the day's portion 12.45 grams of nitrogen; after 16 days of such feeding, the animal was washed, and again after an interval of 18 days. The latter washings contained 0.240 gram of nitrogen equivalent to only 0.013 gram daily.

Analyses of the blood of a sheep on a urea-free ration and on rations containing 20 and 40 grams of urea daily showed that the urea content on the latter rations was considerably above the normal level, the increase being greater with the larger dose of urea. Also urea feeding induced a diuresis and with some animals a temporary change in the color and reaction of the urine; the color became bright yellow and the reaction changed from alkaline to neutral or even acid.

The whole picture of the urea-fed animal is that of a flooding of the tissues with a constituent of no value in nutrition that must be gotten rid of, even to the extent of excreting it by ways not normally utilized to any but an inappreciable extent. The results of Scheunert, Klein, and Steuber thus possess a significance quite contrary to the views of Völtz, while offering some explanation of the results upon which these views were based. In urea feeding experiments, the nitrogen balance of an animal may not possess the significance usually ascribed to it. Furthermore, the success in the feeding of low-protein rations supplemented by urea may be attributable to the favorable effect of urea on digestion and food consumption, and to the gradual attainment of true nitrogen equilibrium on the low nitrogen intake of the basal ration itself. The endogenous nitrogen catabolism of the sheep is evidently very low—approximately 30 mgms. of urinary nitrogen per kgm. of body weight—and may not be attained until several weeks of low-protein feeding.

A final experiment of Scheunert, Klein, and Steuber on two young lambs 4 to 5 months of age on a low-protein ration, one lamb receiving urea and the other not, was not successful in demonstrating any favorable effect of the urea. Both of the animals declined steadily in weight and the experiment had to be terminated at the end of 8 weeks of feeding.

Honcamp and Schneller [183] have also reported indifferent success in

[183] Honcamp, F., and Schneller, E., *Biochem. Z.*, 1923, cxxxviii, 461.

the feeding of urea to growing lambs. Two wethers 21 months of age were placed upon a ration of meadow hay, oat straw and a salt mixture, and, in a second period, upon the same ration plus 10.5 grams of urea daily. The nitrogen balances of these animals were improved to some extent in the urea period, more especially with one of the two lambs, but with this lamb the digestibility of all nutrients was also appreciably higher in the period of urea feeding. In the urea period, the increases in the daily output of urinary nitrogen were equal to 86 and 96 per cent of the added urea nitrogen.

The experiment is interpreted to mean that the synthesis of protein from urea by paunch microörganisms requires more easily soluble carbohydrates than a roughage ration, such as was first used, would afford. Hence, the experiment was repeated with a ration consisting of meadow hay, oat straw and salts with small additions of cracked corn (60 grams) and potato starch flour (90 grams). In this case, the nitrogen balances were considerably improved in the urea period, and the experiment is taken as a clear confirmation of the bacterial-protein theory of urea utilization. However, the urea again favored, though slightly, the digestibility of all nutrients in the ration. In particular, the nitrogen in the feces was distinctly less in the urea period, while the increases in the output of urinary nitrogen in this period were equivalent to 78 and 82 per cent of the urea nitrogen consumed. The addition of casein, equivalent in nitrogen content to the urea dose of 10.5 grams, to either of the two basal rations exerted much greater effects on the nitrogen balances than the urea additions.

Although Honcamp and Schneller interpret the last experiment as a clear indication that protein synthesis from urea requires the presence of readily soluble carbohydrates in the feed, their third experiment complicates the matter still further. In this experiment upon 2 lambs, a practical sheep ration of pea straw and cabbage-turnips was used with and without a urea addition. It was found that urea exerted no effect on the nitrogen balance and was apparently excreted quantitatively in the urine. It follows that protein synthesis from urea requires the presence in the ration not only of readily soluble carbohydrates, but of certain particular soluble carbohydrates. However, these necessary complications in the interpretation of experimental results in terms of the bacterial-protein theory cannot but detract from the credibility of the theory.

The latest work of Morgen and his associates,[184] reported in 1924, yielded entirely negative results on the value of urea as a supplement.

[184] Morgen, A., Windheuser, C., and Ohlmer, E., *Landw. Vers.-Stats.*, 1924, ciii, 1.

either to low-protein or high-protein rations, for lactating sheep and goats. On the other hand, the addition of ammonium acetate to the low-protein ration resulted in considerable, though variable, increases in the secretion of milk solids and particularly milk fat; but since the addition of protein produced no such effect, the ammonium acetate effect can hardly be interpreted in terms of the bacterial-protein theory. The nitrogen balances of the few animals subjected to a metabolism study were not appreciably affected by the urea or ammonium acetate additions, and the whole experiment resulted so contrary to what the authors expected on the basis of earlier experiments that they were quite mystified. While they were not prepared to discard entirely the bacterial protein theory of the utilization of urea, it is evident that their confidence was considerably shaken by the outcome of these experiments.

The value of urea in the fattening of lambs has been investigated by Völtz, Jantzon and Reisch,[185] with what appear to be quite negative results. Three groups of lambs, numbering 5, 5, and 4 lambs to the group, were fed the same amount of a basal ration of mixed hay, turnips and minerals determined by the food consumption of the lambs of Group 1. The lambs of Group 2 received in addition 15 to 20 grams of urea per head per day, and the lambs of Group 3 an amount of peanut cake equivalent in nitrogen content to the urea given to Group 2. At the end of 174 days of feeding, the average total gains were 21.59 kgms., 22.11 kgms. and 27.87 kgms. A digestion and metabolism study with one lamb not on the fattening experiment indicated that the urea addition increased neither the digestibility of the basal ration nor the rate of nitrogen retention on it.

At the end of the fattening experiment the lambs were slaughtered and the carcasses dressed in the usual way. The lambs of Group 1 showed the following dressing percentages: 53.4, 50.4, 51.0, 52.5 and 48.8, averaging 51.3. For Group 2, the dressing percentages were: 53.6, 55.3, 51.4, 52.0 and 51.9, averaging 53.0; while for Group 3, these values were 55.9, 53.9, 52.5 and 56.2, averaging 54.7. Considerable significance is attached by the authors to the greater average dressing percentage of Group 2 as compared with Group 1, and upon this average difference, a favorable effect of urea as a supplement to a protein-poor fattening ration is predicted. However, the variability of the individual percentages in the two groups would seem to preclude any safe conclusion that the average difference was due to anything other than the uncontrolled factors in the experiment. In fact, if the smallest percentage in the first group and the largest in the second, are disregarded, the aver-

[185] Völtz, W., Jantzon, J., and Reisch, E., *Landw. Jahrb.*, 1924, lix, 321.

ages of the remaining four in each group become practically identical, *i.e.*, 51.8 and 52.2, respectively. Hence, the experiment may fairly be considered of purely negative significance. The only certain effect of urea to be seen is its condimental effect in stimulating the appetite. It is said that at all times the urea-fed sheep would have consumed more feed than the sheep on the basal ration alone.

In 1924, the same year in which the report of Völtz, Jantzon and Reisch appeared, Lawrow, Moltschanowa, and Ochotnikowa [186] described the results of a series of metabolism experiments on a young goat subsisting upon rations containing urea in most of the periods, but upon urea-free rations in two of the periods. The investigation proves little, one way or the other, concerning the value of urea as a protein substitute, partly because the animal was a capricious consumer of food and at times was evidently in a patholgical state, but also because the feeding periods were not planned in such a way as to permit any close comparisons between the effects of the same ration with and without urea supplements. In the only comparison possible, that between the second half of the seventh period and the eighth period, the withdrawal of urea from the ration did not materially alter the nitrogen balance after the first two days, which should be considered transitional.

In evaluating the bacterial-protein theory of the utilization by ruminants of urea and other non-protein nitrogenous compounds, it must be realized that, though it is offered as an explanation of many observed experimental facts, it has itself not been directly tested experimentally, to any considerable extent at least. Such investigations as have been made of it have not been impressive in their findings. Morgen, Windheuser and Ohlmer [184] have reported the results of incubating paunch bacteria with solutions of asparagine and of urea. No certain evidence of a utilization of the urea could be obtained, while the asparagine was only slowly utilized. Sjollema and Zande [187] have observed that asparagine and aspartic acid are better sources of nitrogen for paunch bacteria than is urea. Many other experiments may be cited to the effect that urea is not a good source of nitrogen for bacteria.[188]

In explaining the apparent utilization of urea by ruminants, it is assumed that the presence of urea in the paunch greatly stimulates the growth of the paunch flora. Evidence on this point, however, is not commonly cited and, in fact, is difficult to find. Scheunert, Klein, and

[186] Lawrow, B. A., Moltschanowa, O. P., and Ochotnikowa, A. J., *Biochem. Z.*, 1924, cliii, 71.
[187] Sjollema, B., and Zande, J. E., van der, *Verslagen Afdeeling Natuurkunde, Königl. Akad. Wiss., Amsterdam*, 1923, xxxi, 657, *Ber. ges. Physiol.*, 1923, xviii, 356.
[188] Braun, H., Stamatelakis, A., and Kondo, S., *Biochem. Z.*, 1924, cxlv, 381; Kondo, S., *ibid.*, 1924, cliii, 302; *ibid.*, 1925, clv, 148; Rubentschik, L., *Centr. Bakt. Parasitenk*, 1925, Abt. II, lxv, 1.

Steuber,[182] in their experiments on sheep, determined the daily production of methane by one sheep (Sheep Z) both on the basal ration alone and on this ration plus 20 grams of urea daily. On the initial basal ration it was found in one respiration test that the daily production of methane was 20.5 liters. After the addition of the urea, four respiration tests were run during a month, the production of methane being 24.8, 24.2, 26.3 and 23.3 liters per day, respectively. After an increase in the intake of starch, the methane production fell to 20.9 liters, and after a further slight increase, to 18.0 liters. The urea was then withdrawn from the ration, after which a reduction in starch intake was found necessary. A final determination indicated a daily production of 15.5 liters of methane. This series of determinations may be taken to indicate a stimulation of paunch fermentation by urea, but the operation of other determining factors prevents any reliable estimate of the extent of this stimulation. It perhaps is open to question whether this stimulation of fermentation necessarily means a stimulation of bacterial or infusorial growth and multiplication.

It may be expected, if urea, or other non-protein nitrogenous substances, is converted into bacterial protein in the paunch, that some of this protein would escape digestion and appear in the feces. Müller [189] found that the addition of bacterial protein to the ration of a dog did not appreciably change the coefficient of digestibility of nitrogen, which remained at about 84. On the other hand, the cells of a large number of different bacteria were found to be extremely resistant to proteolytic enzymes acting *in vitro,* in experiments reported by Fermi.[190]

A number of German experiments, mainly by Morgen and his associates, have been concerned with the question whether the ingestion by ruminants of non-protein extracts of feeds, or of massive doses of ammonium salts or asparagine, increased the excretion of protein in the feces, as might be expected on the basis of the bacterial protein theory of their utilization. These experiments have been reviewd by Armsby,[164] who summarizes his conclusions as follows:

"On the whole, Morgen's results seem to negative the hypothesis of any considerable formation of indigestible bacterial protein from the non-protein of the feed. As regards ammonium salts and asparagin, they agree in this respect with the investigations already summarized in failing to show any increase of either protein nitrogen or pepsin-insoluble nitrogen in the feces. As regards the extracts of various feedingstuffs, the results also agree with the earlier results in showing an increase of the protein nitrogen, but they also strongly support the conclusion that that increase is largely due to the effect of these extracts in stimulating the formation of metabolic products and in part also to the fact that the extract rations contained more pepsin-insoluble nitrogen relatively than did the rations with which

[189] Müller, M., *Arch. ges. Physiol.,* 1906, cxii, 245.
[190] Fermi, C., *Centr. Bakt.,* Abt. I, 1909, lii, 252.

they were compared. It should be added, however, that practically all of Morgen's experiments show a greater increase of pepsin-insoluble nitrogen in the feces than can thus be accounted for, although it is not yet clear what interpretation is to be placed upon this fact.

"Finally, it should be noted that these negative results neither prove nor disprove the possibility of a formation of *digestible* protein from the non-protein of the feed."

However, it is equally true that these negative results are inconsistent with the theory of the formation of only *partially digestible* protein from non-protein substances, and also that bacterial proteins appear to be of this character. The urea feeding experiments that have been reviewed above, in so far as they involved the collection of excreta, give no indication of a greater output of fecal nitrogen during urea ingestion. In fact, more often than not, just the opposite effect was obtained.

Recently Schwarz [191] has attempted a direct study of the part that microörganisms play in the nutrition of ruminants by examining the paunch contents of cattle for bacteria and infusoria. The method used for this purpose involved extracting with water a weighed amount of material taken directly from the paunch of slaughtered cattle; the extract and washings were then filtered through several layers of filter paper. This filtrate is supposed to contain only water-soluble constituents and bacteria. The latter were separated by filtration through a Reichl bacteria filter. The extracted material is said to contain all of the infusoria, which were separated from the food residues by digestion with pepsin-HCl at 37° C. for 20 hours. The infusoria are said to be dissolved by this treatment, while the food residues are unaffected. All determinations of the fractions of the paunch contents thus separated were in terms of nitrogen.

From a number of determinations on the paunch contents of cattle that had been fed only hay of a poor quality, it was found that an average of 11.7 per cent of the nitrogen in the paunch contents was contained in the bacteria, while 20 per cent was contained in the infusoria. On the basis of various estimates of the nitrogen content of microörganisms it is concluded that each kilogram of fresh paunch contents contains 7.5 grams of bacteria and 20.8 grams of infusoria. One hundred kilograms would contain 256 grams of protein in these forms, an amount that would seem to be significant as a contribution to the protein requirements of the ruminant. The studies are taken as a confirmation of the views of Völtz.

An application of these methods to the contents of the cecum of the horse has been reported by Schwarz and his associates,[192] leading them

[191] Schwarz, C., *Biochem. Z.*, 1925, clvi, 130.

[192] Schwarz, C., and Bienert, G., *Arch. ges. Physiol.*, 1926, ccxiii, 556; Schwarz and Tanzer, J., *ibid.*, 1926, ccxiii, 563; Schwarz and Erben, A., *ibid.*, 1926, ccxiii, 571.

to believe that the cecum of the horse, like the paunch of the ruminant, is a protein factory, in which the resident microörganisms form considerable amounts of protein from the soluble nitrogenous substances traversing these segments of the alimentary canal. A similar examination of the contents of the colon and rectum of the horse gave results that are interpreted to mean that the infusorial protein elaborated in the cecum, if not the bacterial protein, is in some way digested and absorbed in the cecum or colon, although no active proteolysis has ever been detected in these organs.

However, the methods of Schwarz cannot go unchallenged. It appears improbable that the extracted material from paunch contents that goes through a paper filter would not contain material other than bacteria that would be retained by a Reichl filter. Still more improbable is it that only the infusorial protein in the extracted material will be dissolved by pepsin-HCl. Many investigations may be cited in which the peptic digestion of hays dissolves large proportions of the contained nitrogen. Morgen, Beger and Westhauser [193] may be cited in particular, since they found in *in vitro* experiments that digestion of roughages with pepsin dissolved from 78 to 80 per cent of the nitrogen, and that a succeeding digestion with trypsin dissolved but little more. Until the uncertainties thus indicated in the method of Schwarz are removed by a searching critical examination in the laboratory, the results obtained with it cannot be considered credible; in any case, they do not bear directly on the question at issue, *i.e.*, the *daily* production of bacterial protein in the paunch.

In conclusion, it may be said that the bacterial-protein theory of the utilization of non-protein substances in ruminants is still in the controversial stage. Probably the microörganisms of the paunch may utilize these substances for growth; the question at issue, is the purely practical one of whether the amount of protein thus formed will ever be appreciable in comparison with the protein requirements of the animal. The question of the digestibility and the biological value of bacterial and infusorial proteins is also involved. Until these questions are approached by experimental methods affording a more direct attack and susceptible of a more rigorous control than the methods heretofore used, little progress can be expected in their solution.

[193] Morgen, A., Beger, C., and Westhauser, F., *Landw. Vers.-Stats.*, 1914, lxxv, 1.

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